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## LEAFY COTYLEDON1 GENES AND THEIR USES

### FIELD OF THE INVENTION

The present invention is directed to plant genetic engineering. In particular, it relates to new embryo-specific genes useful in improving agronomically important plants.

### BACKGROUND OF THE INVENTION

Embryogenesis in higher plants is a critical stage of the plant life cycle in which the primary organs are established. Embryo development can be separated into two main phases: the early phase in which the primary body organization of the embryo is laid down and the late phase which involves maturation, desiccation and dormancy. In the early phase, the symmetry of the embryo changes from radial to bilateral, giving rise to a hypocotyl with a shoot meristem surrounded by the two cotyledonary primordia at the apical pole and a root meristem at the basal pole. In the late phase, during maturation the embryo achieves its maximum size and the seed accumulates storage proteins and lipids. Maturation is ended by the desiccation stage in which the seed water content decreases rapidly and the embryo passes into metabolic quiescent state. Dormancy ends with seed germination, and development continues from the shoot and the root meristem regions.

The precise regulatory mechanisms which control cell and organ differentiation during the initial phase of embryogenesis are largely unknown. The plant hormone abscisic acid (ABA) is thought to play a role during late embryogenesis, mainly in the maturation stage by inhibiting germination during embryogenesis (Black, M. (1991). In *Absciscic Acid: Physiology and Biochemistry*, W. J. Davies and H. G. Jones, eds. (Oxford: Bios Scientific Publishers Ltd.), pp. 99-124) Koornneef, M., and Karssen, C. M. (1994). In *Arabidopsis*, E. M. Meyerowitz and C. R. Somerville, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 313-334). Mutations which effect seed development and are ABA insensitive have been identified in *Arabidopsis* and maize. The ABA insensitive (*abi3*) mutant of *Arabidopsis* and the viviparous1 (*vp1*) mutant of maize are detected mainly during late embryogenesis (McCarty, et al., (1989) *Plant Cell* 1, 523-532 and Parcy et al., (1994) *Plant Cell* 6, 1567-1582). Both the *VP1* gene and the *ABI3* genes have been isolated and were found to share conserved regions

(Giraudat, J. (1995) *Current Opinion in Cell Biology* 7:232-238 and McCarty, D. R. (1995). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46:71-93). The VP1 gene has been shown to function as a transcription activator (McCarty, et al., (1991) *Cell* 66:895-906). It has been suggested that ABI3 has a similar function.

5                   Another class of embryo defective mutants involves three genes: LEAFY COTYLEDON1 and 2 (LEC1, LEC2) and FUSCA3 (FUS3). These genes are thought to play a central role in late embryogenesis (Baumlein, et al. (1994) *Plant J.* 6:379-387; Meinke, D. W. (1992) *Science* 258:1647-1650; Meinke et al., *Plant Cell* 6:1049-1064; West et al., (1994) *Plant Cell* 6:1731-1745). Like the abi3 mutant, leafy cotyledon-type  
10 mutants are defective in late embryogenesis. In these mutants, seed morphology is altered, the shoot meristem is activated early, storage proteins are lacking and developing cotyledons accumulate anthocyanin. As with abi3 mutants, they are desiccation intolerant and therefore die during late embryogenesis. Nevertheless, the immature mutants  
15 embryos can be rescued to give rise to mature and fertile plants. However, unlike abi3 when the immature mutants germinate they exhibit trichomes on the adaxial surface of the cotyledon. Trichomes are normally present only on leaves, stems and sepals, not cotyledons. Therefore, it is thought that the leafy cotyledon type genes have a role in specifying cotyledon identity during embryo development.

                  Among the above mutants, the lec1 mutant exhibits the most extreme  
20 phenotype during embryogenesis. For example, the maturation and postgermination programs are active simultaneously in the lec1 mutant (West et al., 1994), suggesting a critical role for LEC1 in gene regulation during late embryogenesis.

                  In spite of the recent progress in defining the genetic control of embryo development, further progress is required in the identification and analysis of genes  
25 expressed specifically in the embryo and seed. Characterization of such genes would allow for the genetic engineering plants with a variety of desirable traits. For instance, modulation of the expression of genes which control embryo development may be used to alter traits such as accumulation of storage proteins in leaves and cotyledons.  
Alternatively, promoters from embryo or seed-specific genes can be used to direct  
30 expression of desirable heterologous genes to the embryo or seed. The present invention addresses these and other needs.

## SUMMARY OF THE INVENTION

The present invention is based, in part, on the isolation and characterization of LEC1 genes. The invention provides isolated nucleic acid molecules comprising a LEC1 polynucleotide sequence, typically about 630 nucleotides in length, which specifically hybridizes to SEQ ID NO:1 under stringent conditions. The LEC1 polynucleotides of the invention can encode a LEC1 polypeptide of about 210 amino acids, typically as shown in SEQ ID NO:2.

The invention provides an isolated nucleic acid molecule comprising a LEC1 polynucleotide sequence, the polynucleotide sequence defined as follows: the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under stringent conditions; or the polynucleotide sequence has at least 70% sequence identity to SEQ ID NO:1. In alternative embodiments, the isolated nucleic acid molecule of can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity to SEQ ID NO:1; and, the isolated nucleic acid molecule can have the sequence set forth in SEQ ID NO:1.

In different embodiments, the isolated nucleic acid molecule of the invention is a LEC1 polynucleotide between about 100 nucleotides and about 630 nucleotides in length, and, between about 50 and about 210 amino acids in length. The isolated nucleic acid molecule of the invention can encode a LEC1 polypeptide having an amino acid sequence as shown in SEQ ID NO: 2.

The invention also provides an isolated LEC1 nucleic acid molecule further comprising an operably linked promoter. In alternative embodiments, the promoter is a constitutive promoter, where the constitutive promoter is a cauliflower mosaic virus (CaMV) 35S transcription initiation region or a 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and the promoter is an inducible promoter. The promoter can also be a plant promoter. In various embodiments, the plant promoter is a tissue-specific promoter and is a tissue-specific promoter active in vegetative tissue or reproductive tissue. The plant promoter can be from a LEC1 gene, where LEC1 gene can be as shown in SEQ ID NO:3. In different embodiments, the plant promoter can be from about nucleotide 1 to about nucleotide 1998 of SEQ ID NO:3, or, the promoter can be from the LEC1 gene is as shown in SEQ ID NO:4. The LEC1 polynucleotide can be linked to the promoter in an antisense orientation.

The invention also provides LEC1 polynucleotide further comprising an expression vector, an expression cassette, or a plant virus.

The invention further provides an isolated nucleic acid molecule comprising a LEC1 polynucleotide sequence, wherein the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under stringent conditions, or (b) the polynucleotide sequence has at least 70% sequence identity to SEQ ID NO:1, and, (ii) wherein the polynucleotide sequence encodes a LEC1 polypeptide of between about 50 and about 210 amino acids. The LEC1 polypeptide can have an amino acid sequence as shown in SEQ ID NO:2. In alternative embodiments, the polynucleotide sequence can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity to SEQ ID NO:1; and, the polynucleotide sequence can have the sequence set forth in SEQ ID NO:1.

The invention provides a transgenic plant comprising a heterologous LEC1 polynucleotide operably linked to a promoter, the LEC1 polynucleotide sequence defined as follows: the polynucleotide sequence specifically hybridizes to SEQ ID NO: 1 under stringent conditions; or the polynucleotide sequence has at least 70% sequence identity to SEQ ID NO:1. In alternative embodiments, the polynucleotide sequence can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity to SEQ ID NO:1; and, the polynucleotide sequence can have the sequence set forth in SEQ ID NO:1. The heterologous LEC1 polynucleotide can encode a LEC1 polypeptide. The LEC1 polypeptide can be SEQ ID NO:2. In the transgenic plant, the heterologous LEC1 polynucleotide can be linked to the promoter in an antisense orientation. The promoter can be from a LEC1 gene. The LEC1 gene can have the sequence as shown in SEQ ID NO:3. The transgenic plant can be a member of the genus Brassica.

The invention provides an isolated LEC1 polypeptide comprising a polypeptide sequence defined as follows: (i) the polypeptide sequence is encoded by a polynucleotide sequence which specifically hybridizes to SEQ ID NO:1 under stringent conditions, or (i) the polypeptide sequence is encoded by a polynucleotide sequence having at least 70% sequence identity to SEQ ID NO:1, or (ii) the polypeptide sequence has at least 50% sequence identity to SEQ ID NO:2. In alternative embodiments, the polynucleotide sequence can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity to SEQ ID NO:1; and, the polynucleotide sequence can have the sequence set forth in SEQ ID NO:1. In alternative embodiments, the isolated LEC1

polypeptide can have at least 60% sequence identity, at least 70% sequence identity, at least 80% sequence identity, at least 90% sequence identity, and at least 95% sequence identity to SEQ ID NO:2. The isolated LEC1 polypeptide can have the sequence set forth in SEQ ID NO:2.

5           The invention also provides a method of modulating seed development in a plant, the method comprising introducing into the plant a heterologous LEC1 polynucleotide operably linked to a promoter, wherein the LEC1 polynucleotide has a sequence defined as follows: the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under stringent conditions; or the polynucleotide sequence has at least 70%  
10           sequence identity to SEQ ID NO:1. In alternative embodiments, the polynucleotide sequence can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity to SEQ ID NO:1; and, the polynucleotide sequence can have the sequence set forth in SEQ ID NO:1.

15           In this method, the heterologous LEC1 polynucleotide can encode a LEC1 polypeptide. The LEC1 polypeptide can have an amino acid sequence as shown in SEQ ID NO:2. The heterologous LEC1 polynucleotide can be linked to the promoter in an antisense orientation. In this method, the heterologous LEC1 polynucleotide can be SEQ ID NO:1. The promoter can be from a LEC1 gene. The method LEC1 gene can be a  
20           sequence as shown in SEQ ID NO:3. The plant can be a member of the genus Brassica. The heterologous LEC1 polynucleotide can be introduced into the plant through a sexual cross. In this method, the heterologous LEC1 polynucleotide can be co-expressed with a second heterologous polynucleotide. The second heterologous nucleotide can be selected from the group consisting of AP2 and RAP2 genes of Arabidopsis, and the second  
25           heterologous polynucleotide can be expressed in the antisense orientation.

          The invention further provides a method of inducing ecotopic development of embryonic tissue in a plant, the method comprising introducing into the plant a heterologous LEC1 polynucleotide operably linked to a promoter, wherein the LEC1 polynucleotide has a sequence defined as follows: the polynucleotide sequence  
30           specifically hybridizes to SEQ ID NO:1 under stringent conditions; or the polynucleotide sequence has at least 70% sequence identity to SEQ ID NO:1. In alternative embodiments, the polynucleotide sequence can have at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence

identity, at least 95% sequence identity to SEQ ID NO:1; and, the polynucleotide sequence can have the sequence set forth in SEQ ID NO:1.

5 In this method, the heterologous LEC1 polynucleotide can be co-expressed with a second heterologous polynucleotide. The second heterologous nucleotide can be selected from the group consisting of AP2 and RAP2 genes of Arabidopsis, and the second heterologous nucleotide can be expressed in the antisense orientation.

10 The invention provides an isolated nucleic acid molecule comprising a plant promoter that specifically hybridizes to a polynucleotide sequence consisting of nucleotides 1 to 1998 of SEQ ID NO:3. The plant promoter sequence can consist essentially of about nucleotides 1 to about 1998 of SEQ ID NO:3, or it can be a subsequence of SEQ ID NO:4. In one embodiment, a polynucleotide sequence can be operably linked to the plant promoter of the invention. The polynucleotide sequence operably linked to the plant promoter can encode a polypeptide, and this polynucleotide sequence can be linked to the promoter in an antisense orientation.

15 The invention also provides a transgenic plant comprising a LEC1 promoter operably linked to a heterologous polynucleotide sequence, wherein the LEC1 promoter has a polynucleotide sequence defined as follows: the polynucleotide sequence specifically hybridizes to SEQ ID NO:3 under stringent conditions; or the polynucleotide sequence specifically hybridizes to SEQ ID NO:4 under stringent conditions. In  
20 alternative embodiments, the heterologous polynucleotide sequence encodes a desired polypeptide, and the heterologous polynucleotide sequence is linked to the LEC1 promoter in an antisense orientation. The LEC1 promoter can have the sequence shown in SEQ ID NO:3 or SEQ ID NO:4. The heterologous polynucleotide sequence can be linked to the LEC1 promoter in an antisense orientation. The transgenic plant can be a  
25 member of the genus Brassica.

The invention further provides a method of targeting expression of a polynucleotide to a seed, the method comprising introducing into a plant a LEC1 promoter operably linked to a heterologous polynucleotide sequence, wherein the LEC1 promoter specifically hybridizes to a polynucleotide sequence consisting of nucleotides 1  
30 to 1998 of SEQ ID NO: 3. In this method, the heterologous polynucleotide sequence can encode a desired polypeptide. The heterologous polynucleotide sequence can be linked to the promoter in an antisense orientation.



## Definitions

The phrase "nucleic acid" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Nucleic acids may also include modified nucleotides that permit correct read through by a polymerase and do not alter expression of a polypeptide encoded by that nucleic acid.

The phrase "polynucleotide sequence" or "nucleic acid sequence" includes both the sense and antisense strands of a nucleic acid as either individual single strands or in the duplex. It includes, but is not limited to, self-replicating plasmids, chromosomal sequences, and infectious polymers of DNA or RNA.

The phrase "nucleic acid sequence encoding" refers to a nucleic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both the full length nucleic acid sequences as well as non-full length sequences derived from the full length sequences. It should be further understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell.

The term "promoter" refers to a region or sequence determinants located upstream or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Such promoters need not be of plant origin, for example, promoters derived from plant viruses, such as the CaMV35S promoter, can be used in the present invention.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, flowers, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the method of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants, as well as certain lower plants such as algae. It includes plants of a variety of ploidy levels, including polyploid, diploid and haploid.

A polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding

sequence which is different from any naturally occurring allelic variants. As defined here, a modified LEC1 coding sequence which is heterologous to an operably linked LEC1 promoter does not include the T-DNA insertional mutants as described in West et al., The Plant Cell 6:1731-1745 (1994).

5                   A polynucleotide "exogenous to" an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include Agrobacterium-mediated transformation, biolistic methods, electroporation, in planta techniques, and the like. Such a plant containing the exogenous nucleic acid is referred to  
10 here as an R<sub>1</sub> generation transgenic plant. Transgenic plants which arise from sexual cross or by selfing are descendants of such a plant.

                  As used herein an "embryo-specific gene" or "seed specific gene" is a gene that is preferentially expressed during embryo development in a plant. For purposes of this disclosure, embryo development begins with the first cell divisions in the zygote and  
15 continues through the late phase of embryo development (characterized by maturation, desiccation, dormancy), and ends with the production of a mature and desiccated seed. Embryo-specific genes can be further classified as "early phase-specific" and "late phase-specific". Early phase-specific genes are those expressed in embryos up to the end of embryo morphogenesis. Late phase-specific genes are those expressed from maturation  
20 through to production of a mature and desiccated seed.

                  A "LEC1 polynucleotide" is a nucleic acid sequence comprising (or consisting of) a coding region of about 100 to about 900 nucleotides, sometimes from about 300 to about 630 nucleotides, which hybridizes to SEQ ID NO:1 under stringent conditions (as defined below), or which encodes a LEC1 polypeptide. LEC1  
25 polynucleotides can also be identified by their ability to hybridize under low stringency conditions (e.g., T<sub>m</sub> -40°C) to nucleic acid probes having a sequence from position 1 to 81 in SEQ ID NO:1 or from position 355 to 627 in SEQ ID NO:1.

                  A "promoter from a LEC1 gene" or "LEC1 promoter" will typically be about 500 to about 2000 nucleotides in length, usually from about 750 to 1500. An  
30 exemplary promoter sequence is shown as nucleotides 1-1998 of SEQ ID NO:3. A LEC1 promoter can also be identified by its ability to direct expression in all, or essentially all, proglobular embryonic cells, as well as cotyledons and axes of a late embryo.

                  A "LEC1 polypeptide" is a sequence of about 50 to about 210, sometimes 100 to 150, amino acid residues encoded by a LEC1 polynucleotide. A full length LEC1

polypeptide and fragments containing a CCAAT binding factor (CBF) domain can act as a subunit of a protein capable of acting as a transcription factor in plant cells. LEC1 polypeptides are often distinguished by the presence of a sequence which is required for binding the nucleotide sequence: CCAAT. In particular, a short region of seven residues (MPIANVI) at residues 34-40 of SEQ ID NO: 3 shows a high degree of similarity to a region that has been shown to required for binding the CCAAT box. Similarly, residues 61-72 of SEQ ID NO: 3 (IQECVSEYISFV) is nearly identical to a region that contains a subunit interaction domain (Xing, et al., (1993) EMBO J. 12:4647-4655).

As used herein, a homolog of a particular embryo-specific gene (e.g., SEQ ID NO:1) is a second gene in the same plant type or in a different plant type, which has a polynucleotide sequence of at least 50 contiguous nucleotides which are substantially identical (determined as described below) to a sequence in the first gene. It is believed that, in general, homologs share a common evolutionary past.

A "polynucleotide sequence from" a particular embryo-specific gene is a subsequence or full length polynucleotide sequence of an embryo-specific gene which, when present in a transgenic plant, has the desired effect, for example, inhibiting expression of the endogenous gene driving expression of an heterologous polynucleotide. A full length sequence of a particular gene disclosed here may contain about 95%, usually at least about 98% of an entire sequence shown in the Sequence Listing, below.

The term "reproductive tissues" as used herein includes fruit, ovules, seeds, pollen, pistols, flowers, or any embryonic tissue.

In the case of both expression of transgenes and inhibition of endogenous genes (e.g., by antisense, or sense suppression) one of skill will recognize that the inserted polynucleotide sequence need not be identical and may be "substantially identical" to a sequence of the gene from which it was derived. As explained below, these variants are specifically covered by this term.

In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional polypeptide, one of skill will recognize that because of codon degeneracy a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the term "polynucleotide sequence from" a particular embryo-specific gene, such as LEC1. In addition, the term specifically includes sequences (e.g., full length sequences) substantially identical (determined as described below) with a LEC1 gene sequence and that encode proteins that retain the function of a LEC1 polypeptide.

In the case of polynucleotides used to inhibit expression of an endogenous gene, the introduced sequence need not be perfectly identical to a sequence of the target endogenous gene. The introduced polynucleotide sequence will typically be at least substantially identical (as determined below) to the target endogenous sequence.

5 Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the sequence is complementary to all or a portion of a reference polynucleotide sequence.

10 Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, 15 BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions 20 (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of 25 comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, at least 80% sequence identity, preferably at least 85%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described 30 herein; preferably BLAST using standard parameters, as described below. Accordingly, LEC1 sequences of the invention include nucleic acid sequences that have substantial identity to SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:4. LEC1 sequences of the invention include polypeptide sequences having substantial identity to SEQ ID NO:2. One of skill will recognize that these values can be appropriately adjusted to determine

corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 40%, preferably at least 60%, more preferably at least 90%, and most preferably at least 95%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other, or a third nucleic acid, under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T<sub>m</sub>) for the specific sequence at a defined ionic strength and pH. The T<sub>m</sub> is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 60°C.

In the present invention, mRNA encoded by embryo-specific genes of the invention can be identified in Northern blots under stringent conditions using cDNAs of the invention or fragments of at least about 100 nucleotides. For the purposes of this disclosure, stringent conditions for such RNA-DNA hybridizations are those which include at least one wash in 0.2X SSC at 63°C for 20 minutes, or equivalent conditions. Genomic DNA or cDNA comprising genes of the invention can be identified using the same cDNAs (or fragments of at least about 100 nucleotides) under stringent conditions, which for purposes of this disclosure, include at least one wash (usually 2) in 0.2X SSC at

a temperature of at least about 50°C, usually about 55°C, for 20 minutes, or equivalent conditions.

### BRIEF DESCRIPTION OF THE DRAWINGS

5                   Figure 1 is a restriction map of the 7.4 kb genomic wild-type fragment shown in SEQ ID NO:4.

                  Figure 2A show a schematic representation of the three domains of the LEC1 polypeptide. Figure 2B shows a comparison of the predicted amino acid sequence of the B domain encoded by LEC1 with HAP3 homologs from maize, chicken, lamprey, 10   Xenopus laevis, human, mouse, rat, Emericella nidulans, Schizosaccharomyces pombe, Saccharomyces cerevisiae, and Kluyveromyces lactis. The DNA-binding region and the subunit interaction region are indicated. Numbers indicate amino acid positions of the B domains.

### 15                   DESCRIPTION OF THE PREFERRED EMBODIMENT

                  The present invention provides new embryo-specific genes useful in genetically engineering plants. Polynucleotide sequences from the genes of the invention can be used, for instance, to direct expression of desired heterologous genes in embryos (in the case of promoter sequences) or to modulate development of embryos or other 20   organs (e.g., by enhancing expression of the gene in a transgenic plant). In particular, the invention provides a new gene from Arabidopsis referred to here as LEC1. LEC1 encodes polypeptides which subunits of a protein which acts as a transcription factor. Thus, modulation of the expression of this gene can be used to manipulate a number of useful traits, such as increasing or decreasing storage protein content in cotyledons or 25   leaves.

                  Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, 30   restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook et al., Molecular Cloning - A Laboratory Manual, 2nd. ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989).

Isolation of nucleic acids of the invention

The isolation of sequences from the genes of the invention may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library from a desired plant species. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a library of embryo-specific cDNAs, mRNA is isolated from embryos and a cDNA library which contains the gene transcripts is prepared from the mRNA.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned embryo-specific gene such as the polynucleotides disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species.

Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology to amplify the sequences of the genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other in vitro amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes.

Appropriate primers and probes for identifying embryo-specific genes from plant tissues are generated from comparisons of the sequences provided herein. For a general overview of PCR see PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990). Appropriate primers for this purpose include, for instance: UP primer - 5' GGA ATT CAG CAA CAA CCC AAC CCC A 3' and LP primer - 5' LP primer - 5' GCT CTA GAC ATA CAA CAC TTT TCC TTA 3'. Alternatively, the following primer pairs can be used: 5' ATG ACC AGC TCA GTC ATA GTA GC 3' and 5' GCC ACA CAT GGT GGT TGC TGC TG 3' or 5' GAG ATA GAG ACC GAT CGT GGT TC 3' and 5' TCA CTT ATA CTG ACC ATA ATG GTC 3'. The amplifications conditions are typically as follows. Reaction components: 10 mM Tris-HCl, pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.001% gelatin, 200 microM (uM) dATP, 200 microM dCTP, 200 microM dGTP, 200 microM dTTP, 0.4 microM primers, and 100 units per ml Taq

polymerase. Program: 96 C for 3 min., 30 cycles of 96 C for 45 sec., 50 C for 60 sec., 72 for 60 sec, followed by 72 C for 5 min.

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers et al., Cold Spring Harbor Symp. Quant. Biol. 47:411-418 (1982), and Adams et al., J. Am. Chem. Soc. 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

#### 10 Analysis of LEC1 Gene Sequences

The genus of LEC1 nucleic acid sequences of the invention includes genes and gene products identified and characterized by analysis using the sequences nucleic acid sequences, including SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:4, and protein sequences, including SEQ ID NO:2. LEC1 sequences of the invention include nucleic acid sequences having substantial identity to SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:4. LEC1 sequences of the invention include polypeptide sequences having substantial identity to SEQ ID NO:2. "Substantial identity" of a sequence means that it comprises a sequence that has at least 70% sequence identity, at least 80% sequence identity, preferably at least 85%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described herein.

Optimal alignment of sequences for comparison can use any means to analyze sequence identity (homology) known in the art, e.g., by the progressive alignment method of termed "PILEUP" (see below); by the local homology algorithm of Smith & Waterman (1981) Adv. Appl. Math. 2: 482; by the homology alignment algorithm of Needleman & Wunsch (1970) J. Mol. Biol. 48:443; by the search for similarity method of Pearson (1988) Proc. Natl. Acad. Sci. USA 85: 2444; by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI); ClustalW (CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, described by, e.g., Higgins (1988) Gene 73: 237-244; Corpet (1988) Nucleic Acids Res. 16:10881-90; Huang (1992) Computer Applications in the Biosciences 8:155-65, and Pearson (1994) Methods in Molec. Biol. 24:307-31), Pfam (Sonnhammer (1998) Nucleic Acids Res. 26:322-325); TreeAlign (Hein (1994) Methods Mol. Biol. 25:349-364; MES-ALIGN, and SAM sequence alignment computer programs;



or, by inspection. See also Morrison (1997) Mol. Biol. Evol. 14:428-441, as an example of the use of PILEUP.

Another example of algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul (1990) J. Mol. Biol. 215: 403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>; see also Zhang (1997) Genome Res. 7:649-656 (1997) for the "PowerBLAST" variation. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, supra.). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The term BLAST refers to the BLAST algorithm which performs a statistical analysis of the similarity between two sequences; see, e.g., Karlin (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability ( $P(N)$ ), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

#### Use of nucleic acids of the invention to inhibit gene expression

The isolated sequences prepared as described herein, can be used to prepare expression cassettes useful in a number of techniques. For example, expression

cassettes of the invention can be used to suppress endogenous LEC1 gene expression. Inhibiting expression can be useful, for instance, in weed control (by transferring an inhibitory sequence to a weedy species and allowing it to be transmitted through sexual crosses) or to produce fruit with small and non-viable seed.

5           A number of methods can be used to inhibit gene expression in plants. For instance, antisense technology can be conveniently used. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The expression cassette is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has  
10       been suggested that antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy et al., Proc. Nat. Acad. Sci. USA, 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340.

          The nucleic acid segment to be introduced generally will be substantially  
15       identical to at least a portion of the endogenous embryo-specific gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene.

20           For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of  
25       between about 30 or 40 nucleotides and about full length nucleotides should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 500 nucleotides is especially preferred.

          Catalytic RNA molecules or ribozymes can also be used to inhibit  
30       expression of embryo-specific genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within

antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Haseloff et al. Nature, 334:585-591 (1988).

Another method of suppression is sense suppression. Introduction of expression cassettes in which a nucleic acid is configured in the sense orientation with respect to the promoter has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al., The Plant Cell 2:279-289 (1990), and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184.

Generally, where inhibition of expression is desired, some transcription of the introduced sequence occurs. The effect may occur where the introduced sequence contains no coding sequence per se, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

For sense suppression, the introduced sequence in the expression cassette, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants which are overexpressers. A higher identity in a shorter than full length sequence compensates for a longer, less identical sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and

identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used.

Another means of inhibiting LEC1 function in a plant is by creation of dominant negatives. In this approach, non-functional, mutant LEC1 polypeptides, which retain the ability to interact with wild-type subunits are introduced into a plant.

Identification of residues that can be changed to create a dominant negative can be determined by published work examining interaction of different subunits of CBF homologs from different species (see, e.g., Sinha et al., (1995). Proc. Natl. Acad. Sci. USA 92:1624-1628.)

#### Use of nucleic acids of the invention to enhance gene expression

Isolated sequences prepared as described herein can also be used to prepare expression cassettes which enhance or increase endogenous LEC1 gene expression. Where overexpression of a gene is desired, the desired gene from a different species may be used to decrease potential sense suppression effects. Enhanced expression of LEC1 polynucleotides is useful, for example, to increase storage protein content in plant tissues. Such techniques may be particularly useful for improving the nutritional value of plant tissues.

One of skill will recognize that the polypeptides encoded by the genes of the invention, like other proteins, have different domains which perform different functions. Thus, the gene sequences need not be full length, so long as the desired functional domain of the protein is expressed. As explained above, LEC1 polypeptides are related to CCAAT box-binding factor (CBF) proteins. CBFs are highly conserved family of transcription factors that regulate gene activity in eukaryotic organisms (see, e.g., Mantvani (1992) Nucl. Acids Res. 20:1087-1091; Li (1992) Nucleic Acids Res. 20:1087-1091). LEC1 was found to have high similarity to a portion of the HAP3 subunit of CBF. HAP3 is divided into three domains, an amino terminal A domain, a central B domain, and a carboxyl terminal C domain, as shown diagrammatically in Figure 2A. Specifically, LEC1, has between about 75% and 85% sequence similarity, which is equivalent to 55% to 63% sequence identity, with the B domains of the other HAP3 homologs shown in Figure 2B; see also, Example 1, below. Figure 2B shows the amino acid sequence homology between LEC1 and other CBF homologs.

The LEC1 polypeptide also has an amino terminal A domain, a central B domain, and a carboxyl terminal C domain. The three domains of the LEC1 polypeptide

are defined as follows: in SEQ ID NO:2, the A domain is located between about amino acid position 1 to about position 27; the B domain is located between about amino acid position 28 to about position 117; and, the C domain is located between about position 118 to about position 208. For the corresponding LEC1 encoding LEC1 nucleotide sequence of SEQ ID NO 1: the A domain is located between about nucleotide position 1 to about nucleotide position 82; the B domain is located between about nucleotide position 83 to about nucleotide position 351; the C domain is located between about nucleotide position 352 to about nucleotide position 624.

The DNA binding activity, and, therefore, transcription activation function, of LEC1 polypeptides is thought to be modulated by a short region of seven residues (MPLANVI) at residues 34-40 of SEQ ID NO: 2. Thus, the polypeptides of the invention will often retain these sequences.

Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described for instance, in Sambrook et al., supra. Hydroxylamine can also be used to introduce single base mutations into the coding region of the gene (Sikorski, et al., (1991). Meth. Enzymol. 194: 302-318). For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

Desired modified LEC1 polypeptides can be identified using assays to screen for the presence or absence of wild type LEC1 activity. Such assays can be based on the ability of the LEC1 protein to functionally complement the hap3 mutation in yeast. As noted above, it has been shown that homologs from different species functionally interact with yeast subunits of the CBF. (Sinha, et al., (1995). Proc. Natl. Acad. Sci. USA 92:1624-1628); see, also, Becker, et al., (1991). Proc. Natl. Acad. Sci. USA 88: 1968-1972). The reporter for this screen can be any of a number of standard reporter genes such as the lacZ gene encoding beta-galactosidase that is fused with the regulatory DNA sequences and promoter of the yeast CYC1 gene. This promoter is regulated by the yeast CBF.

A plasmid containing the LEC1 cDNA clone is mutagenized in vitro according to techniques well known in the art. The cDNA inserts are excised from the plasmid and inserted into the cloning site of a yeast expression vector such as pYES2 (Invitrogen). The plasmid is introduced into hap3- yeast containing a lacZ reporter that is

regulated by the yeast CBF such as pLG265UP1-lacZ (Guarente, et al., (1984) Cell 36: 317-321). Transformants are then selected and a filter assay is used to test colonies for beta-galactosidase activity. After confirming the results of activity assays, immunochemical tests using a LEC1 antibody are performed on yeast lines that lack  
5 beta-galactosidase activity to identify those that produce stable LEC1 protein but lack activity. The mutant LEC1 genes are then cloned from the yeast and their nucleotide sequence determined to identify the nature of the lesions.

In other embodiments, the promoters derived from the LEC1 genes of the invention can be used to drive expression of heterologous genes in an embryo-specific or  
10 seed-specific manner, such that desired gene products are present in the embryo, seed, or fruit. Suitable structural genes that could be used for this purpose include genes encoding proteins useful in increasing the nutritional value of seed or fruit. Examples include genes encoding enzymes involved in the biosynthesis of antioxidants such as vitamin A, vitamin C, vitamin E and melatonin. Other suitable genes encoding proteins involved in  
15 modification of fatty acids, or in the biosynthesis of lipids, proteins, and carbohydrates. Still other genes can be those encoding proteins involved in auxin and auxin analog biosynthesis for increasing fruit size, genes encoding pharmaceutically useful compounds, and genes encoding plant resistance products to combat fungal or other infections of the seed.

Typically, desired promoters are identified by analyzing the 5' sequences  
20 of a genomic clone corresponding to the embryo-specific genes described here. Sequences characteristic of promoter sequences can be used to identify the promoter. Sequences controlling eukaryotic gene expression have been extensively studied. For instance, promoter sequence elements include the TATA box consensus sequence  
25 (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G (or T) N G. J. Messing et al., in Genetic Engineering in Plants, pp. 221-227 (Kosage, Meredith and  
30 Hollaender, eds. (1983)).

A number of methods are known to those of skill in the art for identifying and characterizing promoter regions in plant genomic DNA (see, e.g., Jordano, et al., Plant Cell, 1: 855-866 (1989); Bustos, et al., Plant Cell, 1:839-854 (1989); Green, et al.,

EMBO J. 7, 4035-4044 (1988); Meier, et al., Plant Cell, 3, 309-316 (1991); and Zhang, et al., Plant Physiology 110: 1069-1079 (1996)).

#### Preparation of recombinant vectors

5 To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising et al. Ann. Rev. Genet. 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a  
10 cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for overexpression, a plant promoter fragment may be  
15 employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of  
20 *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill.

Alternatively, the plant promoter may direct expression of the polynucleotide of the invention in a specific tissue (tissue-specific promoters) or may be otherwise under more precise environmental control (inducible promoters). Examples of  
25 tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues, such as fruit, seeds, or flowers. As noted above, the promoters from the LEC1 genes described here are particularly useful for directing gene expression so that a desired gene product is located in embryos or seeds. Other suitable promoters include those from genes encoding storage proteins or the lipid body  
30 membrane protein, oleosin. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light.

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences (e.g., promoters or coding regions) from genes of the invention will typically comprise a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

LEC1 nucleic acid sequences of the invention are expressed recombinantly in plant cells to enhance and increase levels of endogenous LEC1 polypeptides. Alternatively, antisense or other LEC1 constructs (described above) are used to suppress LEC1 levels of expression. A variety of different expression constructs, such as expression cassettes and vectors suitable for transformation of plant cells can be prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, e.g., Weising et al. Ann. Rev. Genet. 22:421-477 (1988). A DNA sequence coding for a LEC1 polypeptide, e.g., a cDNA sequence encoding a full length protein, can be combined with cis-acting (promoter) and trans-acting (enhancer) transcriptional regulatory sequences to direct the timing, tissue type and levels of transcription in the intended tissues of the transformed plant. Translational control elements can also be used.

The invention provides a LEC1 nucleic acid operably linked to a promoter which, in a preferred embodiment, is capable of driving the transcription of the LEC1 coding sequence in plants. The promoter can be, e.g., derived from plant or viral sources. The promoter can be, e.g., constitutively active, inducible, or tissue specific. In construction of recombinant expression cassettes, vectors, transgenics, of the invention, a different promoters can be chosen and employed to differentially direct gene expression, e.g., in some or all tissues of a plant or animal.

Typically, desired promoters are identified by analyzing the 5' sequences of a genomic clone corresponding to the embryo-specific genes described here. Sequences characteristic of promoter sequences can be used to identify the promoter. Sequences controlling eukaryotic gene expression have been extensively studied. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site.



In most instances the TATA box is required for accurate transcription initiation. In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G (or T) N G. J. Messing et al., in *Genetic Engineering in Plants*, pp. 221-227 (Kosage, Meredith and Hollaender, eds. (1983)). A number of methods are known to those of skill in the art for identifying and characterizing promoter regions in plant genomic DNA (see, e.g., Jordano, et al., *Plant Cell*, 1: 855-866 (1989); Bustos, et al., *Plant Cell*, 1:839-854 (1989); Green, et al., *EMBO J.* 7, 4035-4044 (1988); Meier, et al., *Plant Cell*, 3, 309-316 (1991); and Zhang (1996) *Plant Physiology* 110:1069-1079).

### Constitutive Promoters

A promoter fragment can be employed which will direct expression of LEC1 nucleic acid in all transformed cells or tissues, e.g. as those of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation.

Promoters that drive expression continuously under physiological conditions are referred to as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include those from viruses which infect plants, such as the cauliflower mosaic virus (CaMV) 35S transcription initiation region (see, e.g., Dagless (1997) *Arch. Virol.* 142:183-191); the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens* (see, e.g., Mengiste (1997) *supra*; O'Grady (1995) *Plant Mol. Biol.* 29:99-108); the promoter of the tobacco mosaic virus; the promoter of Figwort mosaic virus (see, e.g., Maiti (1997) *Transgenic Res.* 6:143-156); actin promoters, such as the *Arabidopsis* actin gene promoter (see, e.g., Huang (1997) *Plant Mol. Biol.* 1997 33:125-139); alcohol dehydrogenase (Adh) gene promoters (see, e.g., Millar (1996) *Plant Mol. Biol.* 31:897-904); and, other transcription initiation regions from various plant genes known to those of skill. See also Holtorf (1995) "Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*," *Plant Mol. Biol.* 29:637-646.

### Inducible Promoters

Alternatively, a plant promoter may direct expression of the LEC1 nucleic acid of the invention under the influence of changing environmental conditions or developmental conditions. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, drought, or the presence of light. Such promoters are referred to herein as "inducible" promoters. For example, the invention incorporates the drought-inducible promoter of maize (Busk (1997) *supra*); the cold, drought, and high salt inducible promoter from potato (Kirch (1997) *Plant Mol. Biol.* 33:897-909).

Alternatively, plant promoters which are inducible upon exposure to plant hormones, such as auxins, are used to express the nucleic acids of the invention. For example, the invention can use the auxin-response elements E1 promoter fragment (AuxREs) in the soybean (*Glycine max* L.) (Liu (1997) *Plant Physiol.* 115:397-407); the auxin-responsive *Arabidopsis* GST6 promoter (also responsive to salicylic acid and hydrogen peroxide) (Chen (1996) *Plant J.* 10: 955-966); the auxin-inducible parC promoter from tobacco (Sakai (1996) 37:906-913); a plant biotin response element (Streit (1997) *Mol. Plant Microbe Interact.* 10:933-937); and, the promoter responsive to the stress hormone abscisic acid (Sheen (1996) *Science* 274:1900-1902).

Plant promoters which are inducible upon exposure to chemicals reagents which can be applied to the plant, such as herbicides or antibiotics, are also used to express the nucleic acids of the invention. For example, the maize In2-2 promoter, activated by benzenesulfonamide herbicide safeners, can be used (De Veylder (1997) *Plant Cell Physiol.* 38:568-577); application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. LEC1 coding sequence can also be under the control of, e.g., a tetracycline-inducible promoter, e.g., as described with transgenic tobacco plants containing the *Avena sativa* L. (oat) arginine decarboxylase gene (Masgrau (1997) *Plant J.* 11:465-473); or, a salicylic acid-responsive element (Stange (1997) *Plant J.* 11:1315-1324).

### Tissue-Specific Promoters

Alternatively, the plant promoter may direct expression of the polynucleotide of the invention in a specific tissue (tissue-specific promoters). Tissue specific promoters are transcriptional control elements that are only active in particular cells or tissues at specific times during plant development, such as in vegetative tissues or

reproductive tissues. Promoters from the LEC1 genes of the invention are particularly useful for tissue-specific direction of gene expression so that a desired gene product is generated only or preferentially in zygotes, embryos or seeds, as described below.

Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only (or primarily only) in certain tissues, such as vegetative tissues, e.g., roots or leaves, or reproductive tissues, such as fruit, ovules, seeds, pollen, pistils, flowers, or any embryonic tissue. Reproductive tissue-specific promoters may be, e.g., ovule-specific, embryo-specific, endosperm-specific, integument-specific, seed and seed coat-specific, pollen-specific, petal-specific, sepal-specific, or some combination thereof.

Suitable seed-specific promoters are derived from the following genes: MAC1 from maize, Sheridan (1996) Genetics 142:1009-1020; Cat3 from maize, GenBank No. L05934, Abler (1993) Plant Mol. Biol. 22:10131-1038; viviparous-1 from Arabidopsis, Genbank No. U93215; atmyc1 from Arabidopsis, Urao (1996) Plant Mol. Biol. 32:571-57; Conceicao (1994) Plant 5:493-505; napA from Brassica napus, GenBank No. J02798, Josefsson (1987) JBL 26:12196-1301; the napin gene family from Brassica napus, Sjodahl (1995) Planta 197:264-271.

The ovule-specific BEL1 gene described in Reiser (1995) Cell 83:735-742, GenBank No. U39944, can also be used. See also Ray (1994) Proc. Natl. Acad. Sci. USA 91:5761-5765. The egg and central cell specific FIE1 promoter is also a useful reproductive tissue-specific promoter.

Sepal and petal specific promoters are also used to express LEC1 nucleic acids in a reproductive tissue-specific manner. For example, the Arabidopsis floral homeotic gene APETALA1 (AP1) encodes a putative transcription factor that is expressed in young flower primordia, and later becomes localized to sepals and petals (see, e.g., Gustafson- Brown (1994) Cell 76:131-143; Mandel (1992) Nature 360:273-277). A related promoter, for AP2, a floral homeotic gene that is necessary for the normal development of sepals and petals in floral whorls, is also useful (see, e.g., Drews (1991) Cell 65:991-1002; Bowman (1991) Plant Cell 3:749-758). Another useful promoter is that controlling the expression of the unusual floral organs (ufo) gene of Arabidopsis, whose expression is restricted to the junction between sepal and petal primordia (Bossinger (1996) Development 122:1093-1102).

A maize pollen-specific promoter has been identified in maize (Guerrero (1990) Mol. Gen. Genet. 224:161-168). Other genes specifically expressed in pollen are

described, e.g., by Wakeley (1998) *Plant Mol. Biol.* 37:187-192; Ficker (1998) *Mol. Gen. Genet.* 257:132-142; Kulikauskas (1997) *Plant Mol. Biol.* 34:809-814; Treacy (1997) *Plant Mol. Biol.* 34:603-611.

Other suitable promoters include those from genes encoding embryonic storage proteins. For example, the gene encoding the 2S storage protein from *Brassica napus*, Dasgupta (1993) *Gene* 133:301-302; the 2s seed storage protein gene family from *Arabidopsis*; the gene encoding oleosin 20kD from *Brassica napus*, GenBank No. M63985; the genes encoding oleosin A, Genbank No. U09118, and, oleosin B, Genbank No. U09119, from soybean; the gene encoding oleosin from *Arabidopsis*, Genbank No. Z17657; the gene encoding oleosin 18kD from maize, GenBank No. J05212, Lee (1994) *Plant Mol. Biol.* 26:1981-1987; and, the gene encoding low molecular weight sulphur rich protein from soybean, Choi (1995) *Mol Gen, Genet.* 246:266-268, can be used. The tissue specific E8 promoter from tomato is particularly useful for directing gene expression so that a desired gene product is located in fruits.

A tomato promoter active during fruit ripening, senescence and abscission of leaves and, to a lesser extent, of flowers can be used (Blume (1997) *Plant J.* 12:731-746). Other exemplary promoters include the pistil specific promoter in the potato (*Solanum tuberosum* L.) SK2 gene, encoding a pistil-specific basic endochitinase (Ficker (1997) *Plant Mol. Biol.* 35:425-431); the Blec4 gene from pea (*Pisum sativum* cv. Alaska), active in epidermal tissue of vegetative and floral shoot apices of transgenic alfalfa. This makes it a useful tool to target the expression of foreign genes to the epidermal layer of actively growing shoots.

A variety of promoters specifically active in vegetative tissues, such as leaves, stems, roots and tubers, can also be used to express the LEC1 nucleic acids of the invention. For example, promoters controlling patatin, the major storage protein of the potato tuber, can be used, see, e.g., Kim (1994) *Plant Mol. Biol.* 26:603-615; Martin (1997) *Plant J.* 11:53-62. The ORF13 promoter from *Agrobacterium rhizogenes* which exhibits high activity in roots can also be used (Hansen (1997) *Mol. Gen. Genet.* 254:337-343. Other useful vegetative tissue-specific promoters include: the tarin promoter of the gene encoding a globulin from a major taro (*Colocasia esculenta* L. Schott) corm protein family, tarin (Bezerra (1995) *Plant Mol. Biol.* 28:137-144); the curculin promoter active during taro corm development (de Castro (1992) *Plant Cell* 4:1549-1559) and the promoter for the tobacco root-specific gene TobRB7, whose

expression is localized to root meristem and immature central cylinder regions (Yamamoto (1991) Plant Cell 3:371-382).

Leaf-specific promoters, such as the ribulose biphosphate carboxylase (RBCS) promoters can be used. For example, the tomato RBCS1, RBCS2 and RBCS3A genes are expressed in leaves and light-grown seedlings, only RBCS1 and RBCS2 are expressed in developing tomato fruits (Meier (1997) FEBS Lett. 415:91-95). A ribulose biphosphate carboxylase promoters expressed almost exclusively in mesophyll cells in leaf blades and leaf sheaths at high levels, described by Matsuoka (1994) Plant J. 6:311-319, can be used. Another leaf-specific promoter is the light harvesting chlorophyll a/b binding protein gene promoter, see, e.g., Shiina (1997) Plant Physiol. 115:477-483; Casal (1998) Plant Physiol. 116:1533-1538. The Arabidopsis thaliana myb-related gene promoter (Atmyb5) described by Li (1996) FEBS Lett. 379:117-121, is leaf-specific. The Atmyb5 promoter is expressed in developing leaf trichomes, stipules, and epidermal cells on the margins of young rosette and cauline leaves, and in immature seeds. Atmyb5 mRNA appears between fertilization and the 16 cell stage of embryo development and persists beyond the heart stage. A leaf promoter identified in maize by Busk (1997) Plant J. 11:1285-1295, can also be used.

Another class of useful vegetative tissue-specific promoters are meristematic (root tip and shoot apex) promoters. For example, the "SHOOTMERISTEMLESS" and "SCARECROW" promoters, which are active in the developing shoot or root apical meristems, described by Di Laurenzio (1996) Cell 86:423-433; and, Long (1996) Nature 379:66-69; can be used. Another useful promoter is that which controls the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase HMG2 gene, whose expression is restricted to meristematic and floral (secretory zone of the stigma, mature pollen grains, gynoecium vascular tissue, and fertilized ovules) tissues (see, e.g., Enjuto (1995) Plant Cell. 7:517-527). Also useful are kn1-related genes from maize and other species which show meristem-specific expression, see, e.g., Granger (1996) Plant Mol. Biol. 31:373-378; Kerstetter (1994) Plant Cell 6:1877-1887; Hake (1995) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 350:45-51. For example, the Arabidopsis thaliana KNAT1 promoter. In the shoot apex, KNAT1 transcript is localized primarily to the shoot apical meristem; the expression of KNAT1 in the shoot meristem decreases during the floral transition and is restricted to the cortex of the inflorescence stem (see, e.g., Lincoln (1994) Plant Cell 6:1859-1876).

One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue, but may also lead to some expression in other tissues as well.

5 In another embodiment, a LEC1 nucleic acid is expressed through a transposable element. This allows for constitutive, yet periodic and infrequent expression of the constitutively active polypeptide. The invention also provides for use of tissue-specific promoters derived from viruses which can include, e.g., the tobamovirus subgenomic promoter (Kumagai (1995) Proc. Natl. Acad. Sci. USA 92:1679-1683; the  
10 rice tungro bacilliform virus (RTBV), which replicates only in phloem cells in infected rice plants, with its promoter which drives strong phloem-specific reporter gene expression; the cassava vein mosaic virus (CVMV) promoter, with highest activity in vascular elements, in leaf mesophyll cells, and in root tips (Verdaguer (1996) Plant Mol. Biol. 31:1129-1139).

#### 15 Production of transgenic plants

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using  
20 techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host  
25 will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. *Embo J.* 3:2717-2722 (1984).

30 Electroporation techniques are described in Fromm et al. *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein et al. *Nature* 327:70-73 (1987).

*Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See,

for example Horsch et al. Science 233:496-498 (1984), and Fraley et al. Proc. Natl. Acad. Sci. USA 80:4803 (1983).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as seedlessness. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts Isolation and Culture, Handbook of Plant Cell Culture, pp. 124-176, MacMillan Publishing Company, New York, 1983; and Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. Ann. Rev. of Plant Phys. 38:467-486 (1987).

The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera Asparagus, Atropa, Avena, Brassica, Citrus, Citrullus, Capsicum, Cucumis, Cucurbita, Daucus, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Linum, Lolium, Lycopersicon, Malus, Manihot, Majorana, Medicago, Nicotiana, Oryza, Panicum, Parnasetum, Persea, Pisum, Pyrus, Prunus, Raphanus, Secale, Senecio, Sinapis, Solanum, Sorghum, Trigonella, Triticum, Vitis, Vigna, and, Zea. The LEC1 genes of the invention are particularly useful in the production of transgenic plants in the genus Brassica. Examples include broccoli, cauliflower, brussel sprouts, canola, and the like.

#### Use and Recombinant Expression of LEC1 in Combination with other Genes

The LEC1 nucleic acids of the invention can be expressed together with other structural or regulatory genes to achieve a desired effect. A cell or plant, such as a transformed cell or a transgenic plant, can be transformed, engineered or bred to co-express both LEC1 nucleotide and/or LEC1 polypeptide, and another gene or gene product.

The LEC1 nucleic acids of the invention, when expressed in plant reproductive or vegetative tissue, can induce ectopic embryo morphogenesis. Thus, in one embodiment, a LEC1 nucleic acid of the invention is expressed in a sense

conformation in a transgenic plant to induce the expression of ectopic embryo-like structures, as discussed above. In another embodiment, LEC1 is co-expressed with a gene or nucleic acid that increases reproductive tissue mass, e.g., increases fruit size, seed mass, seed protein or seed oils. For example, co-expression of antisense nucleic acid to ADC genes, such as AP2 and RAP2 genes of Arabidopsis, will dramatically increase seed mass, seed protein and seed oils; see, e.g., Jofuku, et al., WO 98/07842; Okamuro (1997) Proc. Natl. Acad. Sci. USA 94:7076-7081; Okamuro (1997) Plant Cell 9:37-47; Jofuku (1994) Plant Cell 6:1211-1225. Thus, co-expression of a LEC1 of the invention, to induce ectopic expression of embryonic cells and tissues, together with another plant nucleic acid and/or protein, such as the seed-mass enhancing antisense AP2 nucleic acid, generates a cell, tissue, or plant (e.g., a transgenic plant) with increased fruit and seed mass, greater yields of embryonic storage proteins, and the like.

In another embodiment, the LEC1 nucleic acids of the invention are expressed in plant reproductive or vegetative cells and tissues which lack the ability to produce functional ADC genes, such as AP2 and RAP2 genes. The LEC1 nucleic acid can be expressed in an ADC "knockout" transgenic plant. Alternatively, the LEC1 nucleic acid can be expressed in a cell, tissue or plant expressing a mutant ADC nucleic acid or gene product. Expression of LEC1 nucleic acid in any of these non-functioning ADC models will also produce a cell, tissue or plant with increased fruit and seed mass, greater yields of embryonic storage proteins, and the like.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

### Example 1

This example describes the isolation and characterization of an exemplary LEC1 gene.

#### Experimental Procedures

##### Plant Material

A lec1-2 mutant was identified from a population of Arabidopsis thaliana ecotype Wassilewskija (Ws-O) lines mutagenized with T-DNA insertions as described before (West et al., 1994). The abi3-3, fus3-3 and lec1-1 mutants were generously



provided by Peter McCourt, University of Toronto and David Meinke, Oklahoma State University. Wild type plants and mutants were grown under constant light at 22°C.

Double mutants were constructed by intercrossing the mutant lines lec1-1, lec1-2, abi3-3, fus3-3, and lec2. The genotype of the double mutants was verified through backcrosses with each parental line. Double mutants were those who failed to complement both parent lines. Homozygous single and double mutants were generated by germinating intact seeds or dissected mature embryos before desiccation on basal media.

#### Isolation and Sequence analysis of Genomic and cDNA Clones

Genomic libraries of Ws-O wild type plants, lec1-1 and lec1-2 mutants were made in GEM11 vector according to the instructions of the manufacturer (Promega). Two silique-specific cDNA libraries (stages globular to heart and heart to young torpedo) were made in ZAPII vector (Stratagene).

The genomic library of lec1-2 was screened using right and left T-DNA specific probes according to standard techniques. About 12 clones that cosegregate with the mutation, were isolated and purified and the entire DNAs were further labeled and used as probes to screen a southern blot containing wild type and lec1-1 genomic DNA. One clone hybridized with plant DNA and was further analyzed. A 7.1 kb XhoI fragment containing the left border and the plant sequence flanking the T-DNA was subcloned into pBluescript-KS plasmid (Stratagene) to form ML7 and sequenced using a left border specific primer (5' GCATAGATGCACTCGAAATCAGCC 3'). The T-DNA organization was partially verified using southern analysis with T-DNA left and right borders and PBR322 probes. The results suggested that the other end of the T-DNA is also composed of left border. This was confirmed by generating a PCR fragment using a genomic plant DNA primer (LP primer 5' GCT CTA GAC ATA CAA CAC TTT TCC TTA 3') and a T-DNA left border specific primer (5' GCTTGGTAATAATTGTCATTAG 3') and sequencing.

The EcoRI insert of ML7 was used to screen a wild type genomic library. Two overlapping clones were purified and a 7.4 EcoRI genomic fragment from the wild type DNA region was subcloned into pBluescript-KS plasmid making WT74. This fragment was sequenced (SEQ ID NO: 4) and was used to screen lec1-1 genomic library and wild type silique-specific cDNA libraries. 8 clones from the lec1-1 genomic library were identified and analyzed by restriction mapping.

From these clones the exact site of the deletion in lec1-1 was mapped and sequenced by amplifying a Xbp PCR fragment using primers (H21 - 5' H21 - 5' CTA

AAA ACA TCT ACG GTT CA 3'; H 17 - 5' TTT GTG GTT GAC CGT TTG GC 3')  
flanking the deletion region in lec1-1 genomic DNA. Clones were isolated from both  
cDNA libraries and partially sequenced. The sequence of the cDNA clones and the wild  
type genomic clone matched exactly, confirming that both derived from the same locus.

- 5 All hybridizations were performed under stringent conditions with 32P random prime  
probes (Stratagene).

Sequencing was done using the automated dideoxy chain termination  
method (Applied Biosystems, Foster City, CA). Data base searches were performed at  
the National Center for Biotechnology Information by using the BLAST network service.

- 10 Alignment of protein sequences was done using PILEUP program (Genetics Computer  
Group, Madison, WI)

#### DNA and RNA blot analysis

- Genomic DNA was isolated from leaves by using the CTAB-containing  
buffer Dellaporta, et al., (1983). Plant Mol. Biol. Reporter 1: 19-21. Two micrograms of  
15 DNA was digested with different restriction endonucleases, electrophoretically separated  
in 1% agarose gel, and transferred to a nylon membrane (Hybond N; Amersham).

- Total RNA was prepared from siliques, two days old seedlings, stems,  
leaves, buds and roots. Poly(A)+ RNA was purified from total RNA by oligo(dT)  
cellulose chromatography, and two micrograms of each Poly(A)+ RNA samples were  
20 separated in 1% denatured formaldehyde-agarose gel. Hybridizations were done under  
stringent conditions unless it specifies otherwise. Radioactive probes were prepared as  
described above.

### Complementation of lec1 mutants

A 3.4 kb Bst<sup>Y</sup>I fragment of genomic DNA (SEQ ID NO: 3) containing sequences from 1.992 kb upstream of the ORF to a region 579 bp downstream from the poly A site was subcloned into the hygromycin resistant binary vector pBIB-Hyg. The LEC1 cDNA was placed under the control of the 35S promoter and the ocs polyadenylation signals by inserting a PCR fragment spanning the entire coding region into the plasmid pART7. The entire regulatory fragment was then removed by digestion with NotI and transferred into the hygromycin resistant binary vector BJ49. The binary vectors were introduced into the Agrobacterium strain GV3101, and constructions were checked by re-isolation of the plasmids and restriction enzyme mapping, or by PCR. Transformation to homozygous lec1-1 and lec1-2 mutants were done using the in planta transformation procedure (Bechtold, et al., (1993). Comptes Rendus de l'Academie des Sciences Serie III Sciences de la Vie, 316: 1194-1199. Dry seeds from lec1 mutants were selected for transformants by their ability to germinate after desiccation on plates containing 5g/ml hygromycin. The transformed plants were tested for the present of the transgene by PCR and by screening the siliques for the present of viable seeds.

### In Situ Hybridization

Experiments were performed as described previously by Dietrich et al. (1989) Plant Cell 1: 73-80. Sections were hybridized with LEC1 antisense probe. As a negative control, the LEC1 antisense probe was hybridized to seed sections of lec1 mutants. In addition, a sense probe was prepared and reacted with the wild type seed sections.

### Results

#### Genetic Interaction Between Leafy Cotyledon-Type Mutants and abi3

In order to understand the genetic pathways which regulate late embryogenesis we took advantage of three Arabidopsis mutants lec2, fus3-3 and abi3-3 that cause similar defects in late embryogenesis to those of lec1-1 or lec1-2. These mutants are desiccation intolerant, sometimes viviparous and have activated shoot apical meristems. The lec2 and fus3-3 mutants are sensitive to ABA and possess trichomes on their cotyledons and therefore can be categorized as leafy cotyledon-type mutants (Meinke et al., 1994). The abi3-3 mutants belong to a different class of late embryo defective mutations that is insensitive to ABA and does not have trichomes on the cotyledons.

The two classes of mutants were crossed to lec1-1 and lec1-2 mutants to construct plants homozygous to both mutations. The lec1 and lec2 mutations interact synergistically, resulting in a double mutant which is arrested in a stage similar to the late heart stage, the double mutant embryo, however, is larger. The lec1 or lec2 and fus3-3 double mutants did not display any epistasis and the resulting embryo had an intermediate phenotype. The lec1/abi3-3 double mutants and lec2/abi3-3 double mutants were ABA insensitive and had a lec-like phenotype. There was no difference between double mutants that consist of either lec1-1 or lec1-2.

No epistasis was seen between the double mutants indicating that each of the above genes, the LEC-type and ABI3 genes, operate in different genetic pathways.

#### LEC1 Functions Early in Embryogenesis

The effects of lec1 is not limited to late embryogenesis, it also has a role in early embryogenesis. The embryos of the lec1/lec2 double mutants were arrested in the early stages of development, while the single mutants developed into mature embryos, suggesting that these genes act early during development.

Further examination of the early stages of the single and double mutations showed defects in the shape, size and cell division pattern of the mutants suspensors. The suspensor of wild type embryo consists of a single file of six to eight cells, whereas the suspensors of the mutants are often enlarged and undergo periclinal divisions. Leafy cotyledon mutants exhibit suspensor anomalies at the globular or transition stage whereas wild type and abi3 mutant do not show any abnormalities.

The number of anomalous suspensors increases as the embryos continue to develop. At the torpedo stage, the wild type suspensor cells undergo programmed cell death, but in the mutants secondary embryos often develop from the abnormal suspensors and, when rescued, give rise to twins.

#### The Organization of the LEC1 Locus in Wild Type Plants and lec1 Mutants

Two mutant alleles of the LEC1 gene have been reported, lec1-1 and lec1-2 (Meinke, 1992; West et al., 1994). Both mutants were derived from a population of plants mutagenized insertionally with T-DNA (Feldmann and Marks, 1987), although lec1-1 is not tagged. The lec1-2 mutant contains multiple T-DNA insertions. A specific subset of T-DNA fragments were found to be closely linked with the mutation. A genomic library of lec1-2 was screened using right and left border T-DNA as probes. Genomic clones containing T-DNA fragments that cosegregate with the mutation were isolated and tested on Southern blots of both wild type and lec1-1 plants. Only one clone

hybridized with Arabidopsis DNA and also gave polymorphic restriction fragment in lec1-1.

The lec1-1 polymorphism resulted from a small deletion, approximately 2 kb in length. Using sequences from the plant fragment flanking the T-DNA, the genomic wild type DNA clones and the lec1-1 genomic clones were isolated. An EcoRI fragment of 7.4 kb of the genomic wild type DNA that corresponded to the polymorphic restriction fragment in lec1-1 was further analyzed and sequenced. The exact site of the deletion in lec1-1 was identified using a PCR fragment that was generated by primers, within the expected borders of the deleted fragment, and sequencing.

In the wild type genomic DNA that corresponded to the lec1-1 deletion, a 626 bp ORF was identified. Southern analysis of wild type DNA and the two mutants DNA probed with the short DNA fragment of the ORF revealed that both the wild type and lec1-2 DNA contain the ORF while the lec1-1 genomic DNA did not hybridize. The exact insertion site of the T-DNA in lec1-2 mutant was determined by PCR and sequencing and it was found that the T-DNA was inserted 115 bp upstream of the ORF's translational initiation codon in the 5'.

In order to isolate the LEC1 gene two cDNA libraries of young siliques were screened using the 7.4 kb DNA fragment as a probe. Seventeen clones were isolated and after further analysis and partial sequencing they were all found to be identical to the genomic ORF. The cDNA contains 626 bp ORF specifying 208 amino acid protein (SEQ ID NO:1 and SEQ ID NO:2).

The LEC1 cDNA was used to hybridize a DNA gel blot containing Ws-O genomic DNA digested with three different restriction enzymes. Using low stringency hybridization we found that there is at least one more gene. This confirmed our finding of two more Arabidopsis ESTs that show homology to the LEC1 gene.

### The LEC1 gene is Embryo Specific

The *lec1* mutants are affected mostly during embryogenesis. Rescued mutants can give rise to homozygous plants that have no obvious abnormalities other than the presence of trichomes on their cotyledons and their production of defective progeny.

5 Therefore, we expected the LEC1 gene to have a role mainly during embryogenesis and not during vegetative growth. To test this assumption poly (A)+ RNA was isolated from siliques, seedling, roots, leaves, stems and buds of wild type plants and from siliques of *lec1* plants. Only one band was detected on northern blots using either the LEC1 gene as a probe or the 7.4 kb genomic DNA fragment suggesting that there is only one gene in the  
10 genomic DNA fragment which is active transcriptionally. The transcript was detected only in siliques containing young and mature embryos and was not detected in seedlings, roots, leaves, stems and buds indicating that the LEC1 gene is indeed embryo specific. In addition, no RNA was detected in siliques of both alleles of *lec1* mutants confirming that this ORF corresponds to the LEC1 gene.

### 15 Expression Pattern of the LEC1 Gene

To study how the LEC1 gene specifies cotyledons identity, we analyzed its expression by in situ hybridization. We specifically focused on young developing embryos since the mutants abnormal suspensors phenotype indicates that the LEC1 gene should be active very early during development.

20 During embryogenesis, the LEC1 transcript was first detected in proglobular embryos. The transcript was found in all cells of the proembryo and was also found in the suspensor and the endosperm. However, from the globular stage and on it accumulates more in the outer layer of the embryo, namely the protoderm and in the outer part of the ground meristem leaving the procambium without a signal. At the torpedo  
25 stage the signal was stronger in the cotyledons and the root meristem, and was more limited to the protoderm layer. At the bent cotyledon stage the signal was present throughout the embryo and at the last stage of development when the embryo is mature and filling the whole seed we could not detect the LEC1 transcript. This might be due to sensitivity limitation and may imply that if the LEC1 transcript is expressed at that stage  
30 it is not localized in the mature embryo, but rather spread throughout the embryo.

### The LEC1 gene encodes a Homolog of CCAAT binding factor.

Comparison of the deduced amino acid sequence of LEC1 to the GenBank reveals significant similarity to a subunit of a transcription factor, the CCAAT box binding factor (CBF). CBFs are highly conserved family of transcription factors that

regulate gene activity in eukaryotic organisms Mantvani, et al., . (1992). Nucl. Acids Res. 20: 1087-1091. They are hetero-oligomeric proteins that consist of between three to four non-homologous subunits. LEC1 was found to have high similarity to CBF-A subunit. This subunit has three domains; A and C which show no conservation between kingdoms and a central domain, B, which is highly conserved evolutionary. Similarly the LEC1 gene is composed of three domains. The LEC1 B domain shares between 75%-85% similarity and 55%-63% identity with different B domains that are found in organisms ranging from yeast to human. Within this central domain, two highly conserved amino acid segments are present. Deletion and mutagenesis analysis in the CBF-A yeast homolog hap3 protein demonstrated that a short region of seven residues (42-48) (LPIANVA) is required for binding the CCAAT box, while the subunit interaction domain lies in the region between residues 69-80 (MQECVSEFISFV) (Xing et al., supra). LEC1 protein shares high homology to those regions.

## 15 DISCUSSION

The lec1 mutant belongs to the leafy cotyledon class that interferes mainly with the embryo program and therefore is thought to play a central regulatory role during embryo development. It was shown before that LEC1 gene activity is required to suppress germination during the maturation stage. Therefore, we analyzed the genetic interaction of homozygous double mutants of the different members of the leafy cotyledon class and the abi3 mutant that has an important role during embryo maturation. All the five different combinations of the double mutants showed either an intermediate phenotype or an additive effect. No epistatic relationship among the four genes was found. These findings suggest that the different genes act in parallel genetic pathways. Of special interest was the double mutant lec1/lec2 that was arrested morphologically at the heart stage, but continued to grow in that shape. This double mutant phenotype indicates that both genes LEC1 and LEC2 are essential for early morphogenesis and their products may interact directly or indirectly in the young developing embryo.

### 25 The Role of LEC1 in Embryogenesis

30 One of the proteins that mediate CCAAT box function, is an heteromeric protein called CBF (also called NFY or CP1). CBF is a transcription activator that regulates constitutively expressed genes, but also participates in differential activation of developmental genes Wingender, E. (1993). Gene Regulation in Eukaryotes (New York: VCH Publishers). In mammalian cells, three subunits have been identified CBF-A,

CBF-B and CBF-C and all of which are required for DNA binding. In yeast, the CBF homolog HAP activates the CYC1 and other genes involved in the mitochondrial electron transport Johnson, et al., *Proteins. Annu. Rev. Biochem.* 58, 799-840. (1989). HAP consists of four subunits hap2, hap3, hap4 and hap5. Only hap2, 3 and 5 are required for DNA binding. CBF-A, B and C show high similarity to the yeast hap3, 2 and 5, respectively. It was also reported that mammalian CBF-A and B can be functionally interchangeable with the corresponding yeast subunits (Sinha et al., *supra.*).

The LEC1 gene encodes a protein that shows more than 75% similarity to the conserved region of CBF-A. CCAAT motifs are not common in plants' promoters and their role in transcription regulation is not clear. However, maize and Brassica homologs have been identified. Search in the Arabidopsis GenBank revealed several ESTs that show high similarity to CBF-A, B and C. Accession numbers of CBF-A (HAP3) homologs: H37368, H76589; CBF-B (HAP2) homologs: T20769; CBF-C (HAP5) homologs: T43909, T44300. These findings and the pleiotropic effects of LEC1 suggest that LEC1 is a member of a heteromeric complex that functions as a transcription factor.

The model suggests that LEC1 acts as transcription activator to several sets of genes, which keep the embryonic program on and repress the germination process. Defective LEC1 expression partially shuts down the embryonic program and as a result the cotyledons lose their embryonic characteristics and the germination program is active in the embryo.

### Example 2

This example demonstrates that LEC1 is sufficient to induce embryonic pathways in transgenic plants.

The phenotype of *lec1* mutants and the gene's expression pattern indicated that LEC1 functions specifically during embryogenesis. A LEC1 cDNA clone under the control of the cauliflower mosaic virus 35S promoter was transferred into *lec1-1* mutant plants in planta using standard methods as described above.

Viable dry seeds were obtained from *lec1-1* mutants transformed with the 35S/LEC1 construct. However, the transformation efficiency was only approximately 0.6% of that obtained normally. In several experiments, half the seeds that germinated (12/23) produced seedlings with an abnormal morphology. Unlike wild type seedlings, these 35S/LEC1 seedlings possessed cotyledons that remained fleshy and that failed to



expand. Roots often did not extend or extended abnormally and sometimes greened. These seedlings occasionally produced a single pair of organs on the shoot apex at the position normally occupied by leaves. Unlike wild type leaves, these organs did not expand and did not possess trichomes. Morphologically, these leaf-like structures more  
5 closely resembled embryonic cotyledons than leaves.

The other 35S/LEC1 seeds that remained viable after drying produced plants that grow vegetatively. The majority of these plants (7) flowered and produced 100% lec1 mutant seeds. Amplification experiments confirmed that the seedlings contained the transgene, suggesting that the 35S/LEC1 gene was inactive in these T2  
10 seeds. No vegetative abnormalities were observed in these plants with the exception that a few displayed defects in apical dominance. A few plants (2) were male sterile and did not produce progeny. One plant that produced progeny segregated 25% mutant Lec1<sup>-</sup> seeds that, when germinated before desiccation and grown to maturity, gave rise to 100% mutant seed, as expected for a single transgene locus. The other 75% of seeds contained  
15 embryos with either a wild type phenotype or a phenotype intermediate between lec1 mutants and wild type. Only 25% of the dry seed from this plant germinated, and all seedlings resembled the embryo-like seedlings described above. Some seedlings continued to grow and displayed a striking phenotype. These 35S/LEC1 plants developed two types of structures on leaves. One type resembled embryonic cotyledons while the  
20 other looked like intact torpedo stage embryos. Thus, ectopic expression of LEC1 induces the morphogenesis phase of embryo development in vegetative cells.

Because many 35S/LEC1 seedlings exhibited embryonic characteristics, the seedlings were analyzed for expression of genes specifically active in embryos. Cruciferin A storage protein mRNA accumulated throughout the 35S/LEC1 seedlings,  
25 including the leaf-like structures. Proteins with sizes characteristic of 12S storage protein cruciferin accumulated in these transgenic seedlings. Thus, 35S/LEC1 seedlings displaying an embryo-like phenotype accumulated embryo-specific mRNAs and proteins. LEC1 mRNA accumulated to a high level in these 35S/LEC1 seedlings in a pattern similar to early stage embryos but not in wild type seedlings. LEC1 is therefore sufficient  
30 to alter the fate of vegetative cells by inducing embryonic programs of development.

The ability of LEC1 to induce embryonic programs of development in vegetative cells establishes the gene as a central regulator of embryogenesis. LEC1 is sufficient to induce both the seed maturation pathway as indicated by the induction of storage protein genes in the 35S/LEC1 seedlings. The presence of ectopic embryos on

leaf surfaces and cotyledons at the position of leaves also shows that LEC1 can activate the embryo morphogenesis pathway. Thus, LEC1 regulates both early and late embryonic processes.

### Example 3

5 This example shows that LEC1 is expressed in zygotes and that the promoters of the invention can therefore be used to target expression in zygotes.

To determine precisely when the LEC1 gene becomes activated, LEC1 RNA levels were analyzed in the egg apparatus of mature female gametophytes before fertilization, in zygotes after fertilization, and in very early stage embryos containing an apical cell and two to three suspensor cells. *In situ* hybridization experiments showed  
10 that LEC1 RNA was present in zygotes and early stage embryos but was not detected in female gametophytes. These results show that the LEC1 promoter becomes active in the zygote.. The LEC1 is therefore useful to target the expression of sense or antisense versions of regulatory genes or cytotoxic genes to zygotes and early stage embryos.

15 The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, databases, Genbank sequences, patents, and patent applications cited herein are hereby incorporated by reference.

20

WHAT IS CLAIMED IS:

1                   1.     An isolated nucleic acid molecule comprising a LEC1  
2 polynucleotide sequence, the polynucleotide sequence defined as follows:  
3                   the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under  
4 stringent conditions; or  
5                   the polynucleotide sequence has at least 70% sequence identity to SEQ ID  
6 NO:1.

1                   2.     The isolated nucleic acid molecule of claim 1, wherein the  
2 polynucleotide sequence has at least 90% sequence identity to SEQ ID NO:1.

1                   3.     The isolated nucleic acid molecule of claim 1, wherein the  
2 polynucleotide sequence is SEQ ID NO:1.

1                   4.     The isolated nucleic acid molecule of claim 1, wherein the LEC1  
2 polynucleotide is between about 100 nucleotides and about 630 nucleotides in length.

1                   5.     The isolated nucleic acid molecule of claim 1, wherein the LEC1  
2 polynucleotide encodes a LEC1 polypeptide of between about 50 and about 210 amino  
3 acids.

1                   6.     The isolated nucleic acid molecule of claim 5, wherein the LEC1  
2 polypeptide has an amino acid sequence as shown in SEQ ID NO: 2.

1                   7.     The isolated nucleic acid molecule of claim 1, further comprising  
2 an operably linked promoter. The isolated nucleic acid molecule of claim 1, further  
3 comprising an operably linked promoter.

1                   8.     The isolated nucleic acid molecule of claim 7, wherein the  
2 promoter is a constitutive promoter.

1                   9.     The isolated nucleic acid molecule of claim 8, wherein the  
2 constitutive promoter is a cauliflower mosaic virus (CaMV) 35S transcription initiation  
3 region.

1                   10.    The isolated nucleic acid molecule of claim 8; wherein the  
2   constitutive promoter is a 1'- or 2'- promoter derived from T-DNA of *Agrobacterium*  
3   *tumefaciens*.

1                   11.    The isolated nucleic acid molecule of claim 7, wherein the  
2   promoter is an inducible promoter.

1                   12.    The isolated nucleic acid molecule of claim 7, wherein the  
2   promoter is a plant promoter.

1                   13.    The isolated nucleic acid molecule of claim 12, wherein the plant  
2   promoter is a tissue-specific promoter.

1                   14.    The isolated nucleic acid molecule of claim 13, wherein the tissue-  
2   specific promoter is active in vegetative tissue.

1                   15.    The isolated nucleic acid molecule of claim 13, wherein the tissue-  
2   specific promoter is active in reproductive tissue.

1                   16.    The isolated nucleic acid molecule of claim 12, wherein the plant  
2   promoter is from a LEC1 gene.

1                   17.    The isolated nucleic acid of claim 16, wherein the plant promoter is  
2   from a LEC1 gene as shown in SEQ ID NO:3.

1                   18.    The isolated nucleic acid of claim 17, wherein the plant promoter is  
2   from about nucleotide 1 to about nucleotide 1998 of SEQ ID NO:3.

1                   19.    The isolated nucleic acid of claim 16, wherein the plant promoter is  
2   from the LEC1 gene is as shown in SEQ ID NO:4.

1                   20.    The isolated nucleic acid of claim 7, wherein the LEC1  
2   polynucleotide is linked to the promoter in an antisense orientation.

1                   21.    The isolated nucleic acid of claim 7, further comprising an  
2   expression vector.

- 1                   22.    An isolated nucleic acid molecule comprising a LEC1  
2 polynucleotide sequence,  
3                   (i)    (a)    wherein the polynucleotide sequence specifically hybridizes  
4 to SEQ ID NO:1 under stringent conditions, or  
5                   (b)    the polynucleotide sequence has at least 70% sequence  
6 identity to SEQ ID NO:1, and  
7                   (ii)   wherein the polynucleotide sequence encodes a LEC1 polypeptide  
8 of between about 50 and about 210 amino acids.
- 1                   23.    The isolated nucleic acid of claim 22, wherein the LEC1  
2 polypeptide has an amino acid sequence as shown in SEQ ID NO:2.
- 1                   24.    A transgenic plant comprising a heterologous LEC1 polynucleotide  
2 operably linked to a promoter, the LEC1 polynucleotide sequence defined as follows:  
3                   the polynucleotide sequence specifically hybridizes to SEQ ID NO: 1  
4 under stringent conditions; or  
5                   the polynucleotide sequence has at least 70% sequence identity to SEQ ID  
6 NO:1.
- 1                   25.    The transgenic plant of claim 24, wherein the heterologous LEC1  
2 polynucleotide encodes a LEC1 polypeptide.
- 1                   26.    The transgenic plant of claim 25, wherein the LEC1 polypeptide is  
2 SEQ ID NO:2.
- 1                   27.    The transgenic plant of claim 24, wherein the heterologous LEC1  
2 polynucleotide is linked to the promoter in an antisense orientation.
- 1                   28.    The transgenic plant of claim 24, wherein the promoter is from a  
2 LEC1 gene.
- 1                   29.    The transgenic plant of claim 28, wherein the LEC1 gene is as  
2 shown in SEQ ID NO:3.
- 1                   30.    The transgenic plant of claim 24, which is a member of the genus  
2 Brassica.

1                   31.     An isolated LEC1 polypeptide comprising a polypeptide sequence  
2     defined as follows:

3                   (i) the polypeptide sequence is encoded by a polynucleotide sequence  
4     which specifically hybridizes to SEQ ID NO:1 under stringent conditions, or

5                   (ii) the polypeptide sequence is encoded by a polynucleotide sequence  
6     having at least 70% sequence identity to SEQ ID NO:1, or

7                   (iii) the polypeptide sequence has at least 50% sequence identity to SEQ  
8     ID NO:2.

1                   32.     The isolated LEC1 polypeptide of claim 31, wherein the  
2     polypeptide sequence has at least 85% sequence identity to SEQ ID NO:2.

1                   33.     The isolated LEC1 polypeptide of claim 32, wherein the  
2     polypeptide sequence has the sequence set forth in SEQ ID NO:2.

1                   34.     The isolated LEC1 polypeptide of claim 31, wherein the  
2     polynucleotide sequence has the sequence set forth in SEQ ID NO:1.

1                   35.     A method of modulating seed development in a plant, the method  
2     comprising introducing into the plant a heterologous LEC1 polynucleotide operably  
3     linked to a promoter, wherein the LEC1 polynucleotide has a sequence defined as  
4     follows:

5                   the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under  
6     stringent conditions; or

7                   the polynucleotide sequence has at least 70% sequence identity to SEQ ID  
8     NO:1.

1                   36.     The method of claim 35, wherein the heterologous LEC1  
2     polynucleotide encodes a LEC1 polypeptide.

1                   37.     The method of claim 36, wherein the LEC1 polypeptide has an  
2     amino acid sequence as shown in SEQ ID NO:2.

1                   38.     The method of claim 35, wherein the heterologous LEC1  
2     polynucleotide is linked to the promoter in an antisense orientation.

- 1                   39.    The method of claim 35, wherein the heterologous LEC1  
2 polynucleotide is SEQ ID NO:1.
- 1                   40.    The method of claim 35, wherein the promoter is from a LEC1  
2 gene.
- 1                   41.    The method of claim 40, wherein the LEC1 gene is as shown in  
2 SEQ ID NO:3.
- 1                   42.    The method of claim 35, wherein the plant is a member of the  
2 genus Brassica.
- 1                   43.    The method of claim 35, wherein the heterologous LEC1  
2 polynucleotide is introduced into the plant through a sexual cross.
- 1                   44.    The method of claim 35, wherein the heterologous LEC1  
2 polynucleotide is co-expressed with a second heterologous polynucleotide.
- 1                   45.    The method of claim 44, wherein the second heterologous  
2 nucleotide is selected from the group consisting of AP2 and RAP2 genes of Arabidopsis,  
3 and the second heterologous polynucleotide is expressed in the antisense orientation.
- 1                   46.    A method of inducing ecotopic development of embryonic tissue in  
2 a plant, the method comprising introducing into the plant a heterologous LEC1  
3 polynucleotide operably linked to a promoter, wherein the LEC1 polynucleotide has a  
4 sequence defined as follows:  
5                   the polynucleotide sequence specifically hybridizes to SEQ ID NO:1 under  
6 stringent conditions; or  
7                   the polynucleotide sequence has at least 70% sequence identity to SEQ ID  
8 NO:1.
- 1                   47.    The method of claim 46, wherein the heterologous LEC1  
2 polynucleotide is co-expressed with a second heterologous polynucleotide.
- 1                   48.    The method of claim 47, wherein the second heterologous  
2 nucleotide is selected from the group consisting of AP2 and RAP2 genes of Arabidopsis,  
3 and the second heterologous nucleotide is expressed in the antisense orientation.

1                   49.     An isolated nucleic acid molecule comprising a plant promoter that  
2 specifically hybridizes to a polynucleotide sequence consisting of nucleotides 1 to --1998  
3 of SEQ ID NO:3.

1                   50.     The isolated nucleic acid molecule of claim 49, wherein the plant  
2 promoter sequence consists essentially of about nucleotides 1 to --about 1998 of SEQ ID  
3 NO:3.

1                   51.     The isolated nucleic acid molecule of claim 49 wherein the plant  
2 promoter sequence is a subsequence of SEQ ID NO:4.

1                   52.     The isolated nucleic acid molecule of claim 49, further comprising  
2 a polynucleotide sequence operably linked to the plant promoter.

1                   53.     The isolated nucleic acid of claim 52, wherein the polynucleotide  
2 sequence operably linked to the plant promoter encodes a polypeptide.

1                   54.     The isolated nucleic acid molecule of claim 53, wherein the  
2 polynucleotide sequence is linked to the promoter in an antisense orientation.

1                   55.     A transgenic plant comprising a LEC1 promoter operably linked to  
2 a heterologous polynucleotide sequence, wherein the LEC1 promoter has a  
3 polynucleotide sequence defined as follows:  
4                   the polynucleotide sequence specifically hybridizes to SEQ ID NO:3 under  
5 stringent conditions; or  
6                   the polynucleotide sequence specifically hybridizes to SEQ ID NO:4 under  
7 stringent conditions.

1                   56.     The transgenic plant of claim 55, wherein the heterologous  
2 polynucleotide sequence encodes a desired polypeptide.

1                   57.     The transgenic plant of claim 55, wherein the heterologous  
2 polynucleotide sequence is linked to the LEC1 promoter in an antisense orientation.

1                   58.     The transgenic plant of claim 55, wherein the LEC1 promoter has  
2 the sequence shown in SEQ ID NO:3 or SEQ ID NO:4.



1                    59.    The transgenic plant of claim 55, which is a member of the genus  
2 Brassica.

1                    60.    A method of targeting expression of a polynucleotide to a seed, the  
2 method comprising introducing into a plant a LEC1 promoter operably linked to a  
3 heterologous polynucleotide sequence, wherein the LEC1 promoter specifically  
4 hybridizes to a polynucleotide sequence consisting of nucleotides 1 to --1998 of SEQ ID  
5 NO: 3.

1                    61.    The method of claim 60, wherein the heterologous polynucleotide  
2 sequence encodes a desired polypeptide.

1                    62.    The method of claim 61, wherein the heterologous polynucleotide  
2 sequence is linked to the promoter in an antisense orientation.

1                    63.    A method of targeting expression of a polynucleotide to a seed, the  
2 method comprising introducing into a plant a LEC1 promoter operably linked to a  
3 heterologous polynucleotide sequence, wherein the LEC1 promoter specifically  
4 hybridizes to a polynucleotide sequence consisting of nucleotides 1 to 1998 of SEQ ID  
5 NO: 3.

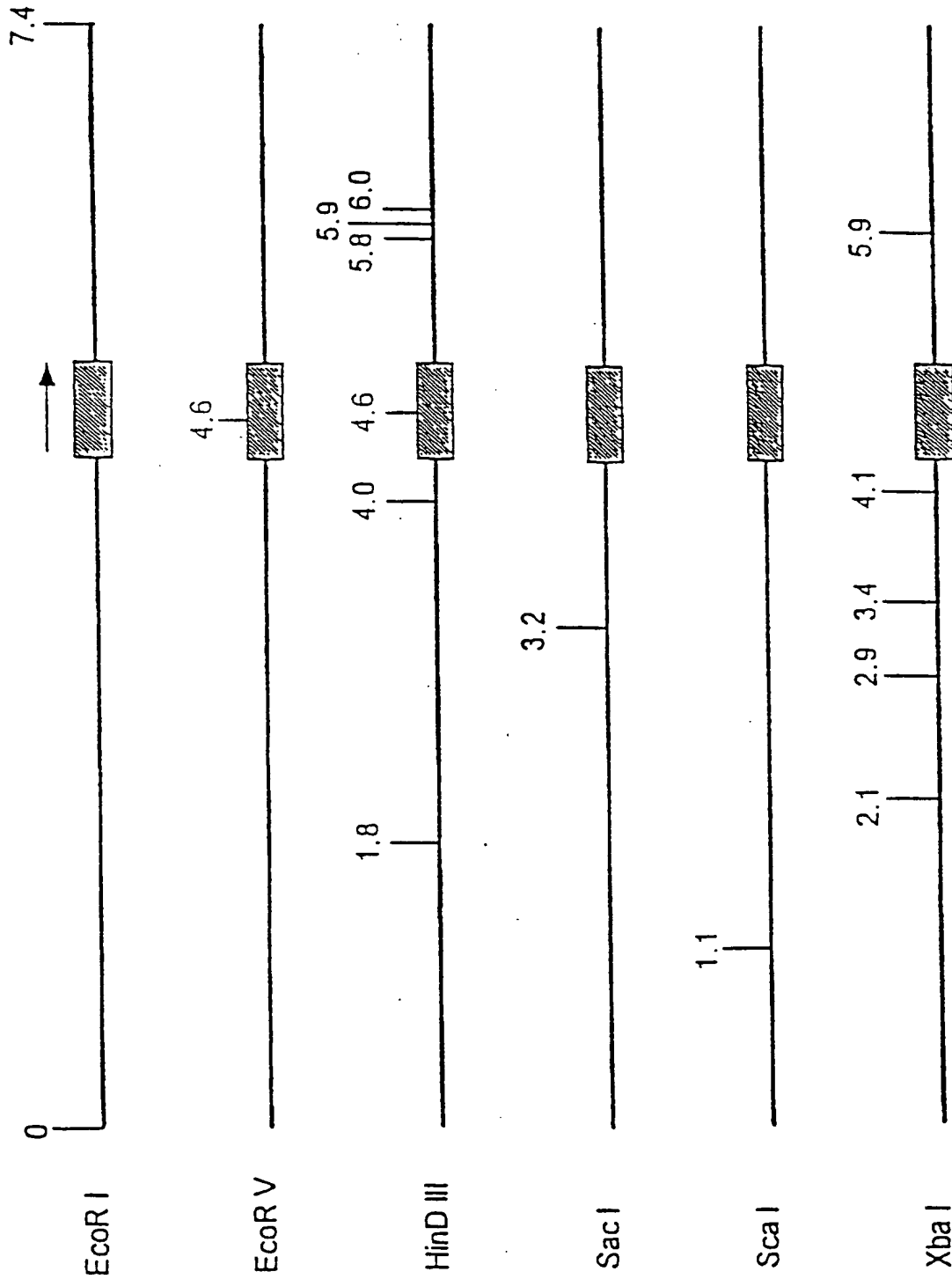
1                    64.    The method of claim 63, wherein the heterologous polynucleotide  
2 sequence encodes a desired polypeptide.

1                    65.    The method of claim 63, wherein the heterologous polynucleotide  
2 sequence is linked to the promoter in an antisense orientation.

1                    66.    The method of claim 63, wherein expression of the heterologous  
2 polynucleotide sequence is targeted to the zygote.

1                    67.    The method of claim 63, wherein expression of the heterologous  
2 polynucleotide sequence is targeted to the embryo.

FIGURE 1



Representative restriction sites in a 7.4 kb EcoR I fragment containing the LEC1 gene. Restriction site positions are given in kilobasepairs. The LEC1 protein coding region is between positions 4.4 kb and 5.05 kb and is indicated by the hatched box.

# Fig. 2

A



B

3

DNA binding

LEC1	28	R	E	Q	D	I	Y	L	P	I	A	N	V	A	R	I	M	K	T	L	P	I	S	H	A	K	I	S	D	D	A	
Maize	30	R	E	Q	D	R	F	L	P	I	A	N	L	S	R	I	M	K	K	A	I	P	I	A	N	G	K	I	A	K	D	A
Chicken	51	R	E	Q	D	I	Y	L	P	I	A	N	V	A	R	I	M	K	N	A	I	P	Q	T	G	K	I	A	K	D	A	
Lamprey	54	R	E	Q	D	I	Y	L	P	I	A	N	V	A	R	I	M	K	T	S	I	P	I	S	S	G	K	I	A	K	D	A
Xenopus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Human	53	R	E	Q	D	I	Y	L	P	I	A	N	V	A	R	I	M	K	N	A	I	P	Q	T	G	K	I	A	K	D	A	
Mouse/Rat	53	R	E	Q	D	I	Y	L	P	I	A	N	V	A	R	I	M	K	N	A	I	P	Q	T	G	K	I	A	K	D	A	
E. nidulans	42	K	E	Q	D	R	W	L	P	I	A	N	V	A	R	I	M	K	L	A	L	P	I	E	N	A	K	I	A	K	E	A
S. pombe	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
S. cerevisiae	38	R	E	Q	D	R	W	L	P	I	N	N	V	A	R	I	M	K	N	T	L	P	P	S	A	K	V	S	K	D	A	
K. lactis	21	A	E	Q	D	R	W	L	P	I	N	N	V	A	R	I	M	K	N	T	L	P	A	T	T	K	V	S	K	D	A	

## Subunit Interaction

LEC1	58	K	E	T	I	Q	E	C	V	S	E	F	I	S	F	V	T	G	E	A	N	F	E	A	C	D	R	E	K	R	K	T
Maize	60	K	E	T	V	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	D	R	E	K	R	K	T	
Chicken	81	K	E	C	V	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	H	Q	E	K	R	K	T	
Lamprey	84	K	E	C	V	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	H	Q	E	K	R	K	T	
Xenopus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Human	83	K	E	C	V	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	H	Q	E	K	R	K	T	
Mouse/Rat	83	K	E	C	V	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	H	Q	E	K	R	K	T	
E. nidulans	72	K	E	C	M	Q	E	C	V	S	E	F	I	S	F	I	T	S	E	A	S	E	A	C	H	Q	E	K	R	K	T	
S. pombe	36	K	E	C	V	Q	E	C	V	S	E	F	I	S	F	V	T	G	E	A	S	E	A	C	H	Q	E	K	R	K	T	
S. cerevisiae	68	K	E	C	M	Q	E	C	V	S	E	F	I	S	F	V	T	G	E	A	S	E	A	C	H	Q	E	K	R	K	T	
K. lactis	51	K	E	C	M	Q	E	C	V	S	E	F	I	S	F	V	T	G	E	A	S	E	A	C	H	Q	E	K	R	K	T	

LEC1	88	I	T	A	E	D	I	L	F	A	M	S	T	L	G	F	O	N	Y	V	D	P	L	T	V	E	L	N	B	Y	A	
Maize	90	I	N	G	E	D	I	L	F	A	M	A	T	L	G	F	E	D	Y	I	E	P	L	K	V	L	O	K	Y	F	A	
Chicken	111	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	O	S	Y	V	E	P	L	K	L	Y	L	O	K	Y	F	
Lamprey	114	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	O	S	Y	V	E	P	L	K	O	Y	L	O	K	Y	F	
Xenopus	28	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	O	S	Y	V	E	P	L	K	L	Y	L	O	K	Y	F	
Human	113	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	O	S	Y	V	E	P	L	K	L	Y	L	O	K	Y	F	
Mouse/Rat	113	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	O	S	Y	V	E	P	L	K	L	Y	L	O	K	Y	F	
E. nidulans	102	V	I	N	G	E	D	I	L	F	A	M	T	S	L	G	F	E	N	Y	A	E	A	L	K	I	Y	L	S	K	Y	F
S. pombe	68	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	E	N	Y	A	E	V	L	K	I	Y	L	S	K	Y	F	
S. cerevisiae	96	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	E	N	Y	A	E	V	L	K	I	Y	L	S	K	Y	F	
K. lactis	81	I	N	G	E	D	I	L	F	A	M	S	T	L	G	F	E	N	Y	A	E	V	L	K	I	Y	L	S	K	Y	F	

Amino Acid Sequence Similarity between LEC1 and Other  
CBF HAP3 Homologs

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Harada, John  
Lotan, Tamar  
Ohto, Masa-aki  
Goldberg, Robert B.  
Fischer, Robert L.
- 10 (ii) TITLE OF INVENTION: Leafy Cotyledon1 Genes and Their Uses
- (iii) NUMBER OF SEQUENCES: 18
- 15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Townsend and Townsend and Crew LLP  
(B) STREET: Two Embarcadero Center, Eighth Floor  
(C) CITY: San Francisco  
(D) STATE: California  
20 (E) COUNTRY: USA  
(F) ZIP: 94111-3834
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
25 (B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
30 (A) APPLICATION NUMBER: US 08/804,534  
(B) FILING DATE: 21-FEB-1997  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
35 (A) NAME: Bastian, Kevin L.  
(B) REGISTRATION NUMBER: 34,774  
(C) REFERENCE/DOCKET NUMBER: 023070-077600US
- (ix) TELECOMMUNICATION INFORMATION:  
40 (A) TELEPHONE: (415) 576-0200  
(B) TELEFAX: (415) 576-0300
- (2) INFORMATION FOR SEQ ID NO:1:
- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 627 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..627

(D) OTHER INFORMATION: /product= "LEC1"

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10 48  
ATG ACC AGC TCA GTC ATA GTA GCC GGC GCC GGT GAC AAG AAC AAT GGT  
Met Thr Ser Ser Val Ile Val Ala Gly Ala Gly Asp Lys Asn Asn Gly  
1 5 10 15

15 96  
ATC GTG GTC CAG CAG CAA CCA CCA TGT GTG GCT CGT GAG CAA GAC CAA  
Ile Val Val Gln Gln Gln Pro Pro Cys Val Ala Arg Glu Gln Asp Gln  
20 25 30

20 144  
TAC ATG CCA ATC GCA AAC GTC ATA AGA ATC ATG CGT AAA ACC TTA CCG  
Tyr Met Pro Ile Ala Asn Val Ile Arg Ile Met Arg Lys Thr Leu Pro  
35 40 45

25 192  
TCT CAC GCC AAA ATC TCT GAC GAC GCC AAA GAA ACG ATT CAA GAA TGT  
Ser His Ala Lys Ile Ser Asp Asp Ala Lys Glu Thr Ile Gln Glu Cys  
50 55 60

30 240  
GTC TCC GAG TAC ATC AGC TTC GTG ACC GGT GAA GCC AAC GAG CGT TGC  
Val Ser Glu Tyr Ile Ser Phe Val Thr Gly Glu Ala Asn Glu Arg Cys  
65 70 75 80

35 288  
CAA CGT GAG CAA CGT AAG ACC ATA ACT GCT GAA GAT ATC CTT TGG GCT  
Gln Arg Glu Gln Arg Lys Thr Ile Thr Ala Glu Asp Ile Leu Trp Ala  
85 90 95

40 336  
ATG AGC AAG CTT GGG TTC GAT AAC TAC GTG GAC CCC CTC ACC GTG TTC  
Met Ser Lys Leu Gly Phe Asp Asn Tyr Val Asp Pro Leu Thr Val Phe  
100 105 110

45 384  
ATT AAC CGG TAC CGT GAG ATA GAG ACC GAT CGT GGT TCT GCA CTT AGA  
Ile Asn Arg Tyr Arg Glu Ile Glu Thr Asp Arg Gly Ser Ala Leu Arg  
115 120 125

50 432  
GGT GAG CCA CCG TCG TTG AGA CAA ACC TAT GGA GGA AAT GGT ATT GGG  
Gly Glu Pro Pro Ser Leu Arg Gln Thr Tyr Gly Gly Asn Gly Ile Gly  
130 135 140

TTT CAC GGC CCA TCT CAT GGC CTA CCT CCT CCG GGT CCT TAT GGT TAT 480

55

Gly Glu Pro Pro Ser Leu Arg Gln Thr Tyr Gly Gly Asn Gly Ile Gly  
130 135 140

5 Phe His Gly Pro Ser His Gly Leu Pro Pro Pro Gly Pro Tyr Gly Tyr  
145 150 155 160

Gly Met Leu Asp Gln Ser Met Val Met Gly Gly Gly Arg Tyr Tyr Gln  
165 170 175

10 Asn Gly Ser Ser Gly Gln Asp Glu Ser Ser Val Gly Gly Gly Ser Ser  
180 185 190

Ser Ser Ile Asn Gly Met Pro Ala Phe Asp His Tyr Gly Gln Tyr Lys  
195 200 205  
15

(2) INFORMATION FOR SEQ ID NO:3:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3395 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30 AGATCCAAAA CAGGTCATGG ACTGGGCCGT AAACCTCTATC CAAAATTCTT  
CATGTTTTTC 60

CATCTTTCAA AAATCTTTAT CCACCATTCC ATTACTAGGG TGTTGGTTTT  
ATTTTATTG 120

35 TTGATTAATT ATGTATTAGA AAATGTAAAG CAATATTCAA TTGTAACATG  
CATCATCTAA 180

40 CACCAATATC TTGTACTAAC CTTTGTAAAT TTTCTATAA ACATTTTAAA  
AGGCTAATT 240

AAATAAAAAT TACAATAAAC GTGATAACTC ACTTTCGTAA CGCATATTTA  
TTCAAATATA 300

45 CCAAAATTTA CCATTTTAAG TAAGAGAATC TTTTAAAAT TAATTTTCAA  
TTTCATTAAT 360

TAAGAAACAA AGAATTTACT GAAACCTATA TTTTATTAAA TTTTAATAAA  
ATATATGACT 420

50 AAAATAACGT CACGTGAATC TTTCTCAGCC GTTCGATAAT CGAATACTTT  
ATTGACTAAG 480

TATTTATTTA GAAAATTTTA AACAACTT AATTTCTAGA AACAAAGAGA  
GCCTCATATG 540  
55

TATAAAAATC TTCTTCTTAT CTTTCTTTCT TTCTTAATAG TCTTTATTTT  
TACTTAATTA 600

5 CTTTGGTAAT TTGTGAAAAA CACAACCAAT GAGAGAAGAG CAGTTTGACT  
GGCCACATAG 660

CCAATGAGAC AAGCCAATGG GAAAGAGATA TAGAGACCTC GTAAGAACCG  
CTCCTTTGCC 720

10 ATTTGTATCA TCTCTCTATA AAACCACTCA ACCATCAACC TNTCTTTGCA  
TGCAACAAAT 780

CACTCAAATA ATTATTTTAT AAAGAACAAA AAAAAAAGA CGGCAGAGAA  
ACAATGGAAC 840

15 GTGGAGCTCC CTTCTCTCAC TATCAGCTAC CCAAATCCAT CTCTGGTAAT  
CTAAGTGGCT 900

ATTGTATAC AGTATATACT TGCCTCCATG TATATTTATA TTCTCGTGAA  
20 AAATTGGAGA 960

CATGCTTTAT GAATTTTATG AGACTTTGCA ACAACGAACG AGATGCTTTC  
TCTCTAGAAA 1020

25 TTAAATTTA GATTTGTGAA GGTTTTGGGA ATGGCCCGGA GAAGACGATT  
TTATATATAC 1080

ATGCATGCAA GAGTTTGATA TGTATATTGT TTCATCATGG CTGAGTCAAA  
GTTTTATCCA 1140

30 AATATTTCCA TGGTGTGGTA TTAGTTAAAC AAATCTCTCG TATGTGTCAT  
TGAATATACC 1200

CGTGCATGTA CCAGGAATGT TTTTGATTCT AAAAACGTTT TTTTCTTTGT  
35 TGTAACGGTT 1260

GAGTTTTTTT CTTCGTTTCA AAACGAGATT CTCGTTTGTC TCTTCCCTTG  
TCTAAAACA 1320

40 TCTACGGTTC ATGTGATTCA AAAACACTAA AAAAATATAA ACTCATTTTT  
TTTAATACT 1380

TAACATTTAA ACTATATATA TATATATATA TATATATATC TTATACTAGT  
CCCAAGTTTT 1440

45 AGTGTGAGGT TTTTTTATTC AAAATCTATC AGTACATTTT TTGGAAAAGA  
ACTAAGTGAA 1500

ATTTTCTCCA AATTTTCCTT TTAATTTGA TTTTAAATT ACTGGATGTC  
50 ATTAACTTTA 1560

ATCTTTTGAT TCTTTCAACG TTTACCATTG GGAACCTTCA CATGAAATAA  
ATGTCTACTT 1620



TATTGAGTCA TACCTTCGTC AACATAAATT AATTGATGTT CTTCTCCAAA  
TTTTGAGTTT 1680

5 TTGGTTTTTC TAATAATCTT AACGAAAGCT TTTTGGTATA CATGTAAAAC  
GTAACGGCAA 1740

GAATCTGAAC AGTCTACTCA ACGGGGTCCA TAAGTCTAGA ATGTAGACCC  
CACAAACTTA 1800  
10 CTCTTATCTT ATTGGTCCGT AACTAAGAAC GTGTCCCTCT GATTCTCTTG  
TTTTCTTCTA 1860

ATTAATTCGT ATCCTACAAA TTAAATTATC ATTTCTACTT CAACTAATCT  
TTTTTTATTT 1920

15 CCTAAAGATT TCAATTTCTC TCTGTATTTT CTATGAACAG AATTGAACTT  
GGACCAGCAC 1980

AGCAACAACC CAACCCCAAT GACCAGCTCA GTCATAGTAG CCGGCGCCGG  
TGACAAGAAC 2040  
20 AATGGTATCG TGGTCCAGCA GCAACCACCA TGTGTGGCTC GTGAGCAAGA  
CCAATACATG 2100

CCAATCGCAA ACGTCATAAG AATCATGCGT AAAACCTTAC CGTCTCACGC  
25 CAAAATCTCT 2160

GACGACGCCA AAGAAACGAT TCAAGAATGT GTCTCCGAGT ACATCAGCTT  
CGTGACCGGT 2220

30 GAAGCCAACG AGCGTTGCCA ACGTGAGCAA CGTAAGACCA TAACTGCTGA  
AGATATCCTT 2280

TGGGCTATGA GCAAGCTTGG GTTCGATAAC TACGTGGACC CCCTCACCGT  
35 GTTCATTAAC 2340

CGGTACCGTG AGATAGAGAC CGATCGTGGT TCTGCACTTA GAGGTGAGCC  
ACCGTCGTTG 2400

40 AGACAAACCT ATGGAGGAAA TGGTATTGGG TTTCACGGCC CATCTCATGG  
CCTACCTCCT 2460

CCGGGTCCTT ATGGTTATGG TATGTTGGAC CAATCCATGG TTATGGGAGG  
TGGTCGGTAC 2520

45 TACCAAAACG GGTCGTCGGG TCAAGATGAA TCCAGTGTTG GTGGTGGCTC  
TTCGTCTTCC 2580

ATTAACGGAA TGCCGGCTTT TGACCATTAT GGTCAGTATA AGTGAAGAAG  
GAGTTATTCT 2640  
50 TCATTTTTAT ATCTATTCAA AACATGTGTT TCGATAGATA TTTTATTTTT  
ATGTCTTATC 2700

55 AATAACATTT CTATATAATG TTGCTTCTTT AAGGAAAAGT GTTGTATGTC  
AATACTTTAT 2760

GAGAAACTGA TTTATATATG CAAATGATTG AATCCAAACT GTTTTGTGGA  
TTAAACTCTA 2820

5 TGCAACATTA TATATTTACA TGATCTAAAG GTTTTGTAAT TCAAAAGCTG  
TCATAGTTAG 2880

AAGATAACTA AACATTGTAG TAACCAAGTT TAATTTACTT TTTTGAGTTT  
ACATAACTAA 2940

10 CCAAGCCAAA AGGTTATAAA ATCTAAATTC GTTGAGTTGT CAAACTTCTG  
AAGATTGCTA 3000

15 TCCTCTTTGA GTTGCTTTCT TTTGGGTGCT TGAGTTTCAT TAGGCTGAGC  
TGACTCGTTG 3060

CTCTCTAGTC TTTCATCTCT GTCTTTTCCA AGGATTCATA ACGTTGGTCG  
CTCTCTGTTT 3120

20 CTGCCTACAC TTCTTCAAGG GATCATTACT GAGGCTAAGA GTTAAAGACC  
TGAACCATGG 3180

TTTTCTGTAA CTGGTTCAAG TTCATTCTCC GGTTATTGTG TGGTTATCTT  
TCGGTTAGAT 3240

25 TGAAACCCAT ATGTTTGCTC TGTTCCTTCT AGTTCCAAGT TTAATTTCCG  
GTTATTGTTT 3300

30 GGCTTTTAA AAGTTTTTAA GGTCTATTCT ATGTAAAGAC TATTCTACGT  
ACGTACATTT 3360

ATCGCAAAAT TGAAAGATTA TAAAAAAAAT TGAAA 3395

35 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 7560 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTNACCCT CACTAAAGGG AACAAAAGCT GGGTACCGGG CCCCCCTCG  
AGGTCGACGG 60

50 TATCGATAAG CTTGATATCG AATTCGTGGC CATTAGACCC ATAACTATAT  
GACGATGTTA 120

55 AAGAGAAAAT AAATCATAAA TAAAATAAGA GTCCTTATCA ATAAACCTAA  
TTGGCTAATT 180

TCAACCTCAA AGAGTAGTAG GAACAGGTAA GGTGAAGCCA AACAGCTCCT  
TTTACAGTTG 240

5 GACCACTAGA GCTGATCTGG CATACAAAGT ATGCTTATTG GGCTGTCACG  
GCCCATCCGC 300

AAAATGTCGT TGGTTACGAA GCATCCACGA CATAGACGGT GCCACATGTT  
AGAAAAGTGT 360

10 TTCGGCGATC AAGATTGTGT CCACATCATT AGACGTCTGA ACTGTCCACG  
TGICTATCAA 420

AGCTGGCGTC AAACATTACG TTTTCGTCGT TTGCGCCTCC TAGTTCACAC  
15 GTGCAACGAA 480

CGCGTGCGAC GTATCAAAAT TGTTAATTTT AGCCATGTAT AAAGAATATC  
TACAAAATTA 540

20 ACCTCAGGAA TATTTTGTGTT TTTTCAATTG AGGCCATAAT ATACNTNCCG  
ATNGAAAAAT 600

TTTNCANCAT ATCNCTAATA TCAAAAAATT ATGATGTTAG TAAACGTAAA  
AAATTTACAC 660

25 AAAATAANTT TCACAAAAC TANNNGGGGAA ATTGGAACAA ANAAAAGACT  
GGTGAGTGAT 720

AAGCGATGAT GGCCGGTGAA TCAGGTAGCC GTCCTACAAC GTGGTTGATT  
30 TTGAGCAAAC 780

TCCTATCTAC TCTTCACACT ATTGGAAATC CCAAAATGTC GTCACACCAT  
AATAATGTGA 840

35 ATTTTGTTAT GGAATTTGAG GGAAACAGTA GATATATGTT TCAACCAGTG  
AAAGTTACCC 900

TCCTTTGGAC ATATCTACGA NAGTAGAAAG TAGAAACATT CACTAAACGT  
GACAACTTTA 960

40 TAAATTTTCT TTTTGTAAC TTTCTTTAGA TTTATTTACG ANAAGAGAAA  
TATAAACGTC 1020

ATGCTAATAA AAAATGCATT ATTTTCTACC ATCTAGCTAG AATATTGATC  
45 AAGTCTTCAC 1080

GTTTTTGTGTT TATCTCTTCT CTCATAGGCA TGTCCACAAA AGGGTAAGTT  
TTACTGGTTC 1140

50 AAAATATTGC ATGAGTACTA CTAAGCTCGT ATAGTTTGAT CTTACTATCA  
TTGCGATGAG 1200

GGTTGTTAGT TTGGAAGAAA TAAGGATTTA TGCAAATGGT AATCATTATG  
TCTGCTATTT 1260

55

AAGAAGTAAA TTATGATGCT TGTTGCGTGA ACATATTAAA TTTGCGAAAA  
ATAAGCAAGG 1320

5 ATACACGAGA GAAGCTCAGA TATTCACGTA ACGATGTTTC ATCTCTTCTC  
ATTGAGGAAA 1380

CATATGGCCA TGATATAGCT AATAAGCCTA CGGGATTGTC NTTTCAACGC  
CGAATCTACC 1440

10 AAAGTGTTC ATCTCTTATT ATATATAGTT TGGTTATTTA AGTAATTAGA  
TGCATCATAA 1500

TCTTTTTTTC TGCCAGTTGT AATGCAGATA AAAATATATT GGTTGTTCTA  
AGGATTGTTT 1560

15 AAACGTGCAT GTGTACAAGT TATTATTTAT ATACTTTCAT CTACATGCGA  
TGCATTATTT 1620

20 ATAATGATAA AACTAAGATT TTTAGTTAAA TTTAATAAAG AGCTTACGAG  
CTACAATTAA 1680

TTAGAAATGG TTGCTCAGAA ATCAGAATAC TATATATGAA AAAAGAAGTT  
GGTATACTTG 1740

25 AAAAAAGAAA AAAGTACTTG AAAAGATGGT AAAAGATATA GAACGAGTAT  
ATATCTTACT 1800

CAAGCACGAT AGAAGTTTGT ATCAAAACAT TGCATTCCAA ACCAATGTTT  
GAAGATGGTC 1860

30 AAAGGTGCTA CTCATGATGT GGTGCGAAGA AGCTTACGAA AAATTCTGCA  
ATGAGAGATA 1920

ACTTTATGGG CTGCTTGTTT AATATATTGA AAATCATGGT AGACAACACC  
AAAGTCTCCT 1980

35 TTACCAGAAG TCATATTTCC TTAACCTCAG AATAAGTAAA TCTTCTAGTT  
TATTATTTGA 2040

40 AAGTTGAGCG TATAATTGCA ATGAAACTTT TACCAATTCA CCGCCTCCTA  
ACTGAGTTGT 2100

TGTATTATCC TATCTCTTTA GCTATCCTTT CCTTGCTCTT GCTCCACCTG  
CATGTGGCCT 2160

45 CTTTATTTAT AATCTCTCTA GATTCTGCTA AAGATGTNTG TTCAAATGG  
TTTATCTTTA 2220

AGGGAAGCAA AGTGAATGGA AACATTTAAA GAAAAAAAAA ACTTTTAGCA  
GAGTTCCATG 2280

50 AGATTTTATA CTGATGATAA CTAAAATAAT CTTATATGCG TAAGATTATT  
TTAGTTCTAA 2340

ACTTCATTTT GAAATGAGAG GTCATTGGCC AGGAAAGATT CAATATTGGT  
TCTTTGTAA 2400

5 TTCTCGTTGG TTTGTTTTTA GTATGGGCTA GATCCAAAAC AGGTCATGGA  
CTGGGCCGTA 2460

AACTCTATCC AAAATTCTTC ATGTTTTTCC ATCTTTCAAA AATCTTTATC  
CACCATTCCA 2520

10 TTAGTAGGGT GTTGGTTTTA TTTTATTGTG TGATTAATTA TGTATTAGAA  
AATGTAAAGC 2580

AATATTCAAT TGTAACATGC ATCATCTAAC ACCAATATCT TGTACTAACC  
TTTTGTAATT 2640

15 TTCCTATAAA CATTTTAAAA GGCTAATTTA AATAAAAATT ACAATAAACG  
TGATAACTCA 2700

CTTTCGTAAC GCATATTTAT TCAAATATAC CAAAATTTAC CATTTTAAGT  
20 AAGAGAATCT 2760

TTTTAAAATT AATTTTCAAT TTCATTAATT AAGAAACAAA GAATTTACTG  
AAACCTATAT 2820

25 TTTATTAAAT TTTAATAAAA TATATGACTA AAATAACGTC ACGTGAATCT  
TTCTCAGCCG 2880

TTCGATAATC GAATACTTTA TTGACTAAGT ATTTATTTAG AAAATTTTAA  
ACAACACTTA 2940

30 ATTTCTAGAA ACAAGAGAG CCTCATATGT ATAAAAATCT TCTTCTTATC  
TTTCTTTCTT 3000

TCTTAATAGT CTTTATTTTT ACTTAATTAC TTTGGTAATT TGTGAAAAAC  
35 ACAACCAATG 3060

AGAGAAGAGC AGTTTGACTG GCCACATAGC CAATGAGACA AGCCAATGGG  
AAAGAGATAT 3120

40 AGAGACCTCG TAAGAACCGC TCCTTTGCCA TTTGTATCAT CTCTCTATAA  
AACCCTCAA 3180

CCATCAACCT NTCTTTGCAT GCAACAAATC ACTCAAATAA TTATTTTATA  
AAGAACAAAA 3240

45 AAAAAAGAC GGCAGAGAAA CAATGGAACG TGGAGCTCCC TTCTCTCACT  
ATCAGCTACC 3300

CAAATCCATC TCTGGTAATC TAAGTGGCTA TTTGTATACA GTATATACTT  
50 GCCTCCATGT 3360

ATATTTATAT TCTCGTGAAA AATTGGAGAC ATGCTTTATG AATTTTATGA  
GACTTTGCAA 3420

CAACGAACGA GATGCTTTCT CTCTAGAAAT TTAAATTTAG ATTTGTGAAG  
GTTTTGGGAA 3480

5 TGGCCCGGAG AAGACGATTT TATATATACA TGCATGCAAG AGTTTGATAT  
GTATATTGTT 3540

TCATCATGGC TGAGTCAAAG TTTTATCCAA ATATTTCAT GGTGTGGTAT  
TAGTTAAACA 3600

10 AATCTCTCGT ATGTGTCATT GAATATACCC GTGCATGTAC CAGGAATGTT  
TTTGATTCTA 3660

AAAACGTTTT TTTCTTTGTT GTAACGGTTG AGTTTTTTTC TTCGTTTCAA  
AACGAGATTC 3720

15 TCGTTTGTCT CTTCCCTTGT CTA AAAACAT CTACGGTTCA TGTGATTCAA  
AAACACTAAA 3780

AAAATATAAA CTCATTTTTT TTTAATACTT AACATTTAAA CTATATATAT  
ATATATATAT 3840

20 ATATATATCT TATACTAGTC CCAAGTTTTA GTGTGAGGTT TTTTATTCA  
AAATCTATCA 3900

25 GTACATTTTT TGGAAAAGAA CTAAGTGAAA TTTTCTCCAA ATTTTCCTTT  
TACTATTGAT 3960

TTTTTAATTA CTGGATGTCA TTAAC TTAA TCTTTTGATT CTTTCAACGT  
TTACCATTGG 4020

30 GAACCTTCAC ATGAAATAAA TGTCTACTTT ATTGAGTCAT ACCTTCGTCA  
ACATAAATTA 4080

ATTGATGTTT TTCTCCAAAT TTTGAGTTTT TGGTTTTTCT AATAATCTTA  
ACGAAAGCTT 4140

35 TTTGGTATAC ATGTAAAACG TAACGGCAAG AATCTGAACA GTCTACTCAA  
CGGGGTCCAT 4200

40 AAGTCTAGAA TG TAGACCCC ACAAACTTAC TCTTATCTTA TTGGTCCGTA  
ACTAAGAACG 4260

TGTCCCTCTG ATTCTCTTGT TTTCTTCTAA TTAATTCGTA TCCTACAAAT  
TTAATTATCA 4320

45 TTTCTACTTC AACTAATCTT TTTTATTTC CTAAAGATTT CAATTTCTCT  
CTGTATTTTC 4380

TATGAACAGA ATTGAACTTG GACCAGCACA GCAACAACCC AACCCCAATG  
ACCAGCTCAG 4440

50 TCATAGTAGC CGGCGCCGGT GACAAGAACA ATGGTATCGT GGTCCAGCAG  
CAACCACCAT 4500

GTGTGGCTCG TGAGCAAGAC CAATACATGC CAATCGCAAA CGTCATAAGA  
ATCATGCGTA 4560

5 AACCTTACC GTCTCACGCC AAAATCTCTG ACGACGCCAA AGAAACGATT  
CAAGAATGTG 4620

TCTCCGAGTA CATCAGCTTC GTGACCGGTG AAGCCAACGA GCGTTGCCAA  
CGTGAGCAAC 4680

10 GTAAGACCAT AACTGCTGAA GATATCCTTT GGGCTATGAG CAAGCTTGGG  
TTCGATAACT 4740

ACGTGGACCC CCTCACCGTG TTCATTAACC GGTACCGTGA GATAGAGACC  
GATCGTGGTT 4800

15 CTGCACTTAG AGGTGAGCCA CCGTCGTTGA GACAAACCTA TGGAGGAAAT  
GGTATTGGGT 4860

TTCACGGCCC ATCTCATGGC CTACCTCCTC CGGGTCCTTA TGGTTATGGT  
ATGTTGGACC 4920

AATCCATGGT TATGGGAGGT GGTCGGTACT ACCAAAACGG GTCGTCGGGT  
CAAGATGAAT 4980

25 CCAGTGTTGG TGGTGGCTCT TCGTCTTCCA TTAACGGAAT GCCGGCTTTT  
GACCATTATG 5040

GTCAGTATAA GTGAAGAAGG AGTTATTCTT CATTTTATA TCTATTCAA  
ACATGTGTTT 5100

30 CGATAGATAT TTTATTTTTA TGTCTTATCA ATAACATTTC TATATAATGT  
TGCTTCTTA 5160

AGGAAAAGTG TTGTATGTCA ATACTTTATG AGAAACTGAT TTATATATGC  
AAATGATTGA 5220

ATCCAAACTG TTTTGTGGAT TAAACTCTAT GCAACATTAT ATATTACAT  
GATCTAAAGG 5280

40 TTTTGTAATT CAAAAGCTGT CATAGTTAGA AGATAACTAA ACATTGTAGT  
AACCAAGTTT 5340

AATTTACTTT TTTGAGTTTA CATAACTAAC CAAGCCAAAA GGTTATAAAA  
TCTAAATTCG 5400

45 TTGAGTTGTC AAACCTTCTGA AGATTGCTAT CCTCTTTGAG TTGCTTTCTT  
TTGGGTGCTT 5460

GAGTTTCATT AGGCTGAGCT GACTCGTTGC TCTCTAGTCT TTCATCTCTG  
TCTTTTCCAA 5520

50 GGATTCATAA CGTTGGTCGC TCTCTGTTTC TGCCTACACT TCTTCAAGGG  
ATCATTACTG 5580

AGGCTAAGAG TTAAAGACCT GAACCATGGT TTTCTGTAAC TGGTTCAAGT  
TCATTCTCCG 5640

5 GTTATTGTGT GGTTATCTTT CGGTTAGATT GAAACCCATA TGTTTGCTCT  
GTTTCTTCTA 5700

GTTCCAAGTT TAATTTCCGG TTATTGTTTG GCTTTTAAA AGTTTTTAAG  
GTCTATTCTA 5760

10 TGTAAGACT ATTCTACGTA CGTACATTTA TCGCAAAATT GAAAGATTAT  
AAAAAAAATT 5820

GAAAGATCCA AAGGAAACCA ATAGATTAAA CTAAAATGTA GTATCCTTTT  
TATCATTTTA 5880

15 GGCTATGTTT TCTTTTAAGA AAGCTTTGGT AGTTAACTCT GTTTAAAAGA  
AAAAAAGAG 5940

ATGCATAAAT TAAATTTAAG TTTCTAGAAC TTTTGGATAA ACATATTAAG  
20 CTAAAGAAAT 6000

TAAACTAAAG GCGTAAATG CAAGCTTGTT ATGCGTTATT GAAAACATTA  
CCTCTAAATT 6060

25 AAATAGCCCA ATATTGAAAA CCTTAAGCTT CTTTGATCCC CTTAACTTGT  
TTGTCCACCA 6120

AGTATTAGTT CATCTCTTAA CACGGCAACT CGAAACGGCA CAATGGACAA  
ACATGGTCTT 6180

30 TCAAAAACCA CTTCCCAATA CATCCATCGT CAAACTCGTG GCCACATGGT  
AAGGTCACCA 6240

CTATTTCTCC CTTTCAAAC TCCTCCAAAC AAATTGTGCA CAACTGGCG  
35 TCAGAGTTGG 6300

ATTTCTTCTT ATTATTATAT ACTTTCCTTG CCAAACGGTC AACCACAAAC  
TTATTTGCCG 6360

40 GTCTAATTAA CTCGATATTA TTGGTGGTCT CATCAAACGA GTCAATCCGA  
GGAGGAGGTG 6420

GAACAATGAC TTACAGTAC ATGTAAACTA ACGTAGCACA AACTGAAGAG  
TCTACCATAG 6480

45 AAATCGACTT ACAGATTCGT TCAGTGAGTT GAGAGTTAGC AATGTCAACA  
TATTGTTCCG 6540

AGAGCCCTGC TGAGTACAAC CATTCAATTCA GTTTTTTCGA GTCATTAGGG  
50 TAGGAGGATA 6600

TGACACCTTC GTAGTCATTG TACGAGAGAA CGAAATTG TGAAGACTA  
ATTGATGTGT 6660



CCGATCTTCG GGCACCTACG CAGATTTTGA ATGATCCAGC ATCTTGTGAT  
TTCGGTTTGA 6720

5 GGTCTATTTT GCCGCCAAAG GATATTTCCG CTTCCATAGC TATCAAAGAG  
AAAGAAAAAT 6780

AGTGAATCCA AGGTTTAGGG TTTCTTTTCT TTGTCTTNCT TATATATAGA  
GGCGCTAGAT 6840

10 TGTATTAAGG ATTATACATA TATATAAGTA ATTGCAATTT GTGAGTTTAT  
CCTTATTCAT 6900

TTTTAATTTT ATTTACCTTT ATTTAGTTGA TATTGTGTCC TTTTCCTAGG  
TAGCATTTCC 6960

15 TTCCATCTGT GTTAATTATT AGCATTTCCT TTCCTTTGTC TTATTTCGCT  
TTATTTCGTA 7020

GGAAGAAATC CTTTATGNAC CCCATCTTGG CTGAGAACTT GAGATGATTT  
TAAATCCTCA 7080

20 AAAATTATTC AATTTATGAT TTCGAAATTG ATATACACTT TATATTTTCT  
CCTAAAAAAC 7140

25 CATATTGTAC TAAGAAAAGT AGAAAACCAG ACTTTTAAAT ATGTTAGATT  
TTAATIGGGT 7200

TCTTAAAGTG TTTTAGCGTT TNACACCGGT TATTCTCAA AATCCAAACT  
CTATAATTAT 7260

30 AGTTTTTAAG TATAAATTAA TCCGGTTGGC CCAATTAGTG GACCGTTTAA  
AGAGTAGACA 7320

CTTTTTTTTT TATATATCGA CTACCATAAA ACTTTAACGA TTAATATTTT  
TGGATAATAA 7380

35 GCGATCGTTT TGAGGCGTCC CAATTTTTTT TGTTCCTTTT TATATGAGAA  
ATGGGTTTAA 7440

40 GAAAAACTGC AATTTTGTCC ATAAAGCTAG TCAGAATTCC TGCAGCCCGG  
GGGATCCACT 7500

AGTTCTAGAG CGGCCGCCAC CGCGGTGGAG CTCCAATTCC CCCTATAGTG  
AGTCGTATTA 7560

45

## (2) INFORMATION FOR SEQ ID NO:5:

- 50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear
- 55 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5 Met Pro Ile Ala Asn Val Ile  
1 5

## (2) INFORMATION FOR SEQ ID NO:6:

10

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

15

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Gln Glu Cys Val Ser Glu Tyr Ile Ser Phe Val  
1 5 10

25

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAATTCAGC AACAACCCAA CCCCA

25

40

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

45

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

50

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTCTAGACA TACAACACTT TTCCTTA

27

55

## (2) INFORMATION FOR SEQ ID NO:9:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- 10 (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

15 ATGACCAGCT CAGTCATAGT AGC 23

## (2) INFORMATION FOR SEQ ID NO:10:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- 25 (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

30 GCCACACATG GTGGTTGCTG CTG 23

## (2) INFORMATION FOR SEQ ID NO:11:

- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- 40 (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

45 GAGATAGAGA CCGATCGTGG TTC 23

## (2) INFORMATION FOR SEQ ID NO:12:

- 50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCACCTTATAC TGACCATAAT GGTC

24

10 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCATAGATGC ACTCGAAATC AGCC

24

25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCTTGGTAAT AATTGTCATT AG

22

40

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

55 CTAAAAACAT CTACGGTTCA

20

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 10 (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

15 TTTGTGGTTG ACCGTTTGGC 20

## (2) INFORMATION FOR SEQ ID NO:17:

## 20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
25 (D) TOPOLOGY: linear

## 25 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

30 Leu Pro Ile Ala Asn Val Ala  
1 5

## 35 (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
40 (C) STRANDEDNESS:  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Gln Glu Cys Val Ser Glu Phe Ile Ser Phe Val  
1 5 10  
50